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13. ABSTRACT (Maximum 200) Nitric Oxide (NO) is a potent bioactive product of many normal cells and certain cancer cells. Recently we have shown that tumor-derived NO promoted tumor growth and metastasis in the C3H/HeJ mouse mammary adenocarcinoma model, and that NO induced by IL-2 therapy was responsible for capillary leakage and compromised antitumor effects of IL-2 therapy. Current proposal was to validate further the stimulatory role of NO in tumor progression, and identify the mechanisms for the above and NO-mediated impediments of antitumor effects of IL-2 therapy. Our results to date have shown the expression of endothelial type (e) NOS by tumor cells within spontaneous as well as transplanted C3H/HeJ mammary tumors is positively correlated with metastasis. Currently we are exploring the effects of downregulating eNOS gene on tumor cell behavior. We have further shown that endogenous as well as induced NO promoted invasiveness of a highly metastatic mammary tumor line C3L5. This was due to an upregulation of the invasion-promoting enzyme matrix metalloprotease (MMP)-2 and a downregulation of the MMP inhibitors TIMP-2 and TIMP-3. Finally, we have shown that NO induced by IL-2 therapy suppressed the activation of antitumor killer cells, which was abrogated by addition of NOS inhibitors. Thus NOS inhibitors may have a valuable role in cancer therapy.				
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## 5. Introduction

This project has been designed to explore the role of nitric oxide (NO) in mammary tumor progression, using a C3H/HeJ mouse mammary tumor model developed in our laboratory. This model employs spontaneous tumors as well as their clones which vary in their ability for spontaneous metastasis.

### Biology of NO

Following the discovery (1) that NO accounts for the full biological activity of a factor initially named "endothelium-derived relaxing factor" (2), produced by endothelial cells and causing vasodilation, research on the biology of NO has grown exponentially for many years. This molecule has since been shown to be produced by many other cells in the body, providing additional physiological functions such as inhibition of platelet aggregation, modulation of neurotransmission and mediation of cytotoxic function of macrophages against microbes, parasites and tumor cells (3-8). Sustained high levels of NO produced at the sites of inflammation can also mediate pathological injuries (9).

NO is produced by the conversion of the amino acid L-arginine to L-citrulline by a family of enzymes known as NO synthases (NOS). Three isoforms of NOS have been identified so far: endothelial type or eNOS is a constitutive form present in endothelial cells, myocardial cells and other cells inclusive of certain tumor cells; neuronal type or nNOS is also a constitutive form present in the central nervous system neurons, cells of the myenteric plexus, skeletal muscle cells, renal, bronchial and pancreatic islet cells as well as in tumors of the central nervous system; inducible type or iNOS is usually induced by certain inflammatory cytokines (e.g. IFN- $\gamma$ , TNF- $\alpha$ ) or bacterial products (e.g. LPS) in macrophages, hepatocytes, chondrocytes, endothelial cells and certain tumor cells (10-14). The constitutive forms are  $\text{Ca}^{++}$  and calmodulin-dependent whereas the inducible form is  $\text{Ca}^{++}$  and calmodulin-independent. Genes for all the isoforms have been cloned in numerous species (15,16) and disrupted in mice to show that none of the disruptions were embryo-lethal but had pathological effects consistent with known biological functions of NO. For example, eNOS knockout mice are hypertensive (17) because of the loss of vaso-relaxant function of NO; iNOS knockout mice are susceptible to infection and show poor macrophage cytotoxicity against parasites and tumor cells (18), consistent with NO-mediated macrophage defense; nNOS knockout mice (19) show hypertrophic pyloric stenosis, consistent with NO-mediated relaxation of pyloric sphincter muscle. nNOS - deficient males, in addition, show abnormal sexual behavior (20) because of aberrant neurotransmission.

NO is a free radical capable of crossing the cell membrane and reacting with other molecules. Most physiological functions of NO are mediated by increases in intracellular cGMP (21,22), whereas antibacterial, antiparasitic and antitumor functions of macrophage-

derived NO have been ascribed to the inhibition of mitochondrial respiration and DNA synthesis in target cells (23).

Constitutive production of NO occurs in cells at low to moderate levels, and the resulting bioactivity is short lived ( $T_{1/2}$  = few seconds) and short-range in nature. On the other hand, induced production of NO can be sustained at high local levels for a longer duration if the inducer molecules, e.g. inflammation-associated cytokines are produced in a protracted manner. This often leads to pathological consequences, resulting from NO reaction products. NO reacts with molecular oxygen, transition metals and superoxide to form intermediates which can cause cellular injury. For example, NO reacts with superoxide to make peroxynitrite, which can cause DNA damage (24).

### **Role of NO in tumor progression**

It has been recognized for some time that chronic NO production is genotoxic and thus potentially carcinogenic (24). Recent studies, including our own (25, appendix 1) have revealed that tumor or host-derived NO can profoundly influence tumor progression in a positive or negative manner depending on the circumstances, and that in a large panel of well-established tumors, which have been examined so far, NO usually promotes tumor progression. Elevated serum NO levels have been observed in many cancer patients (26) indicating that tumor cells or host cells serve as the additional source of NO in these patients. A high expression of active NOS enzymes in tumor cells (27, 28), endothelial cells in tumor vasculature (28) or tumor-infiltrating macrophages (29) has been positively correlated with the degree of malignancy in human cancers involving a number of tissues: cancers of the reproductive tract (uterus, ovary) (27), central nervous system tumors (28) and breast cancer (29). However, the underlying mechanisms remain unexplored. Unexpectedly an inversion of this relationship has been reported for human colonic tumors (30,31); this finding is not consistent with another study reporting that many human colon cancer cell lines exhibited significant NOS activity (32). A positive correlation between NOS expression or NO production and tumor progression has also been detected in experimental tumor models in the mouse (33) and the rat (34).

A direct evidence for a stimulatory role of NO in tumor progression came from our own findings in a murine mammary adenocarcinoma model (35,36) that treatments with either of two NOS inhibitors  $N^G$ -methyl-L Arginine (NMMA) and  $N^G$ -nitro-L-Arginine methyl ester (L-NAME) reduced the growth of the primary tumors and their spontaneous lung metastases in mice transplanted with the C3L5 mammary tumor line (see figures 3, 4, 5, 6, in Appendix 1). Similar findings were reported with L-NAME therapy in a rat colonic adenocarcinoma model (34). In support of these results, engineered expression of iNOS in a human colonic adenocarcinoma line resulted in an increased growth rate and vascularity of tumors following transplantation in nude mice (37). In contrast with these results, engineered overexpression of iNOS in an iNOS deficient murine melanoma line

suppressed tumorigenic and metastatic ability of tumor cells because of NO-mediated cytostasis and apoptosis (38,39). Two explanations may be offered for these apparently conflicting results: First, very high NO levels (such as those produced by the iNOS-transduced murine melanoma line) (38,39) can be detrimental to tumor cell survival; for example the iNOS-overexpressing melanoma line had poor survival in the absence of NOS inhibitors *in vitro* and *in vivo* (39). Second, tumor cells may vary in their susceptibility to NO-mediated cytostasis and apoptosis because of their genetic makeup. For example, it has been suggested that the functional status of the tumor suppressor gene p53 dictates susceptibility (if functional) or resistance (if non-functional) to NO-mediated cytostasis or apoptosis (40,41). This suggestion was based on the following findings: iNOS transfected tumor cell lines fell into two distinct categories. Those expressing functional wild type p53 were vulnerable to NO-mediated cytostasis because of an accumulation p53 protein induced by endogenous NO (40,41). On the other hand, tumor cells in which p53 gene was lost or mutated not only withstood the deleterious effects of endogenous NO, but also exhibited faster growth and vascularity when transplanted *in vivo* (41). Since p53 mutation occurs in nearly half of human cancers (42), it was hypothesized that NO would facilitate tumor progression in a large proportion of well-established human tumors (41). We hypothesize that during the clonal evolution of tumors *in vivo*, high NO producing clones susceptible to NO-mediated injury are deleted and selected against those which are genetically resistant to NO-mediated injury and capable of utilizing NO to their advantage for expression of an aggressive phenotype (25, Appendix 1). Loss of functional p53 gene may represent one of many genetic changes which can possibly result in the above phenotype. Further studies are needed to identify other genotypic markers in tumors for susceptibility or resistance to NO-mediated injury, so that the information can be utilized in therapeutic designs (25).

### **C3H/HeJ mammary tumor model employed in the present project.**

Details of this model are provided in ref. 25 (Appendix 1, an invited review article which is in press). In brief, this model is a combination of spontaneous C3H/HeJ mammary tumors and some of their clonal derivatives produced in our laboratory. Approximately 90% of retired breeder females of this mouse strain spontaneously develop invasive mammary adenocarcinomas with a pseudoglandular architecture, most of which metastasize to the lungs. This is due to insertional mutagenesis of certain cell growth-regulating loci resulting from the integration of the proviral form of the mouse mammary tumor virus (MMTV) in the developing mammary tissue of mice receiving the virus via mother's milk. Approximately 39% of human breast cancer specimens express a 660 bp sequence of the MMTV envelop gene (43), the epidemiological significance of which remain to be identified. This finding and the similarity in histological features suggest that C3H/HeJ spontaneous mammary tumors may represent the closest model for the human breast cancer, in particular, the familial form. We have derived two clonal lines, C3L5 and C10, grown from a spontaneous mammary tumor-derived line T58. The metastatic



phenotype for C3L5 is high, for C10 is low, and for T58 is intermediate, based on the number of spontaneous lung metastases from subcutaneously transplanted tumors. In addition, based on histological evidence of tissue invasiveness of primary tumors, C3L5 is highly invasive and C10 has a low to moderate invasive ability.

Preliminary data provided in the original grant application revealed that spontaneous C3H/HeJ primary tumors expressed eNOS protein (based on immunocytochemistry) in a heterogeneous manner in tumor cells, whereas their metastases in the lungs were uniformly and strongly positive for eNOS. This finding suggested that eNOS bearing cells in the primary tumor were more prone to metastasis. This suggestion was strengthened by the findings that C3L5 cells (highly metastatic) were strongly positive for eNOS *in vitro*, as well as *in vivo* both at primary and metastatic sites. In addition, iNOS was inducible in C3L5 cells when cultured with IFN- $\gamma$  and LPS. In contrast, C10 cells (poorly metastatic) were weakly positive for eNOS, and the expression was heterogeneous. These findings, combined with our published observations (35, 36, see also figures 3, 4, 5 and 6 in Appendix 1) that two NOS inhibitors NMMA and L-NAME reduced the growth of C3L5 primary tumors as well as their spontaneous lung metastases, led us to hypothesize that tumor-derived NO promoted tumor progression in this mammary tumor model. A large component of the current project is to validate this hypothesis and to identify the mechanisms underlying NO-mediated promotion of tumor progression in this model.

### **Role of NO in "capillary leak syndrome"**

We have discovered that capillary leak syndrome (characterized by fluid leakage from the capillaries into tissue spaces, various organs and body cavities), a life-threatening side effect of interleukin-2 (IL-2) based cancer immunotherapy, is due to the increased production of nitric oxide (44, 45). This was shown by (a) a positive correlation of NO levels in the serum and the body fluids with the severity of IL-2 therapy-induced capillary leakage in healthy and tumor-bearing mice, and (b) an amelioration of this capillary leakage by chronic oral administration of NOS inhibitors NMMA and L-NAME (44, 45, 46, 47, see Appendix 2 for a comprehensive summary). Unexpectedly, we also observed that additional therapy with NOS inhibitors improved antitumor/antimetastatic effects of IL-2 therapy (44, 47). This finding led to the suggestion that NO induction by IL-2 therapy interfered with antitumor effects of IL-2 therapy.

A minor component of the current project was to (a) identify the cellular source of NO induced by IL-2 therapy, and (b) identify possible immunological mechanisms underlying NO-mediated interference with antitumor effects of IL-2 therapy.

## **6. BODY OF THE PROGRESS REPORT**

**Overall Hypothesis:** (1) Tumor derived NO promotes C3H/HeJ mammary tumor progression and metastasis, and (2) IL-2 induced NO production compromises antitumor effects of IL-2 therapy.

**Overall Objectives:** (1) To validate the hypothesis of the stimulatory role of NO in mammary tumor progression by further correlation of eNOS expression with metastasis, and investigating the effects of down-regulating eNOS gene on tumor growth, angiogenesis and metastases. (2) To identify mechanisms of NO-mediated stimulation of tumor progression by investigating the role of NO in tumor cell proliferation, invasiveness and angiogenesis. (3) To identify possible immune mechanisms of NO-mediated interference with the antitumor effects of IL-2 therapy.

### **Our assessment of overall progress in relation to the statement of objectives**

- Task 1 Relationship between NOS expression and tumor progression/metastasis:** Progress has matched with our expectations. The molecular biology component has been frustrating at the beginning because of our failure to knockout the eNOS gene in C3L5 cells. We have now started the antisense experiments to downregulate eNOS. Recruitment of a molecular biologist was delayed unexpectedly because of the failure of arrival of the recruited candidate. We have now recruited another experienced biochemist/molecular biologist and the work is proceeding smoothly. The above has resulted in some carryover of the salary line from Year 1, which has been budgeted into the current project (Year II onwards).
- Task 2 Identification of mechanisms of tumor progression by NO.** Although this task was initially assigned to Year II onwards, we have achieved significant progress in this area within Year I.
- Task 3 Mechanisms underlying interference with antitumor effects of IL-2 therapy by IL-2-induced NO.** Although this task was initially assigned to Year III onwards, significant progress has been achieved in this area within Year I. We shall conduct some newer experiments (to complete this task) to be designed on the basis of newer knowledge gathered during the current year. (See later).

## **Record of Research findings during the current year.**

### **Task 1 Relationship between NOS expression and tumor progression and metastasis.**

#### **(a) Relationship between the expression of NOS protein and tumor growth and metastasis.**

**(i) Spontaneous C3H/HeJ mammary tumors.** We have now expanded and validated our preliminary data (presented in the original proposal) in another 5 spontaneous mammary tumors. Since development of spontaneous tumors takes considerable time, we have not yet been able to group a sufficient number of tumors into slow, intermediate and fast growth rate categories in order to examine whether the level of expression of NOS proteins has any bearing to the growth rate of primary tumors and their metastases.

#### **Materials and Methods:**

5 C3H/HeJ spontaneous tumors were harvested from C3H/HeJ retired breeder females at 8-12 weeks of tumor age. Based on the growth rate of primary tumors, one had fast growth rate, three had intermediate growth rates and one had low growth rate. The latter tumor showed some spontaneous regression which occurs in about a quarter of the tumors. All animals showed lung metastases. Histologically, all primary tumors showed local tissue invasiveness, however the low growth rate (regressing) tumor showed evidence of tumor cell death and extensive mononuclear cell infiltration.

Both primary and metastatic tumors (5  $\mu$ m thick sections fixed in buffered formalin and paraffin-embedded) were permeabilized in 0.1% triton in PBS for 20 min and immunostained for eNOS and iNOS enzymes by treatment with primary antibodies (polyclonal eNOS rabbit and monoclonal iNOS mouse antibodies, Transduction Lab, Lexington, KY) followed by biotinylated goat anti-rabbit or horse anti-mouse secondary antibodies and avidin-biotin conjugate (ABC) and DAB chromogen treatment. Negative controls were provided by normal rabbit serum or mouse Ig replacing the primary antibodies.

**Results:** All primary tumors showed tumor cells positive for eNOS. There was a good deal of heterogeneity in the distribution of eNOS positive cells within the same tumor irrespective of growth rate (42-71%, depending on the site within the tumor). The mean % eNOS positive cells did not correlate well with growth rates of tumors. However, all metastatic tumors were

strongly positive for eNOS (75-93% +ve cells). iNOS positivity was never seen in primary or metastatic tumor cells. Only a certain proportion of macrophages and stromal cells showed iNOS staining. A representative micrograph is provided in figure 1 of Appendix 1.

**Conclusions:** Spontaneous primary tumors were heterogenous for eNOS protein expression irrespective of growth rate but tumor cells in metastatic foci were always predominantly eNOS positive. Tumor cells did not express iNOS, but iNOS was expressed by certain macrophages and stromal cells.

These results strengthen our hypothesis that eNOS expression provides a selective advantage for primary tumor cells to metastasize.

**(ii) C3L5 (highly metastatic) and C10 (weakly metastatic) cell lines and their transplants.** We have already shown that 100% of C3L5 cells grown in culture immunostained for eNOS but were negative for iNOS. However, C3L5 cells expressed iNOS (25-30% of cells) when cultured with IFN- $\gamma$  and LPS which served as the iNOS inducer (see figure 2 in Appendix 1). C10 cells were weakly positive for eNOS (heterogenous in expression), and negative for iNOS. We have now conducted eNOS and iNOS staining in another batch of 5 primary tumors and their metastases in the lungs developing in C3H/HeJ female mice at 3-4 weeks after subcutaneous transplantation of  $10^5$  C3L5 cells. The methods were the same as described for spontaneous tumor tissues. The results have confirmed our earlier data. Subcutaneous primary tumors showed eNOS positivity in nearly every tumor cell. Strong eNOS positivity was also seen in the majority of C3L5 tumor cells at the site of spontaneous lung metastasis (see figure 1, Appendix 1).

- (b) Attempts to investigate biological alterations of murine mammary carcinoma cell line(C3L5) caused by downregulation of eNOS gene expression in these cells.**

#### **Methods and Results:**

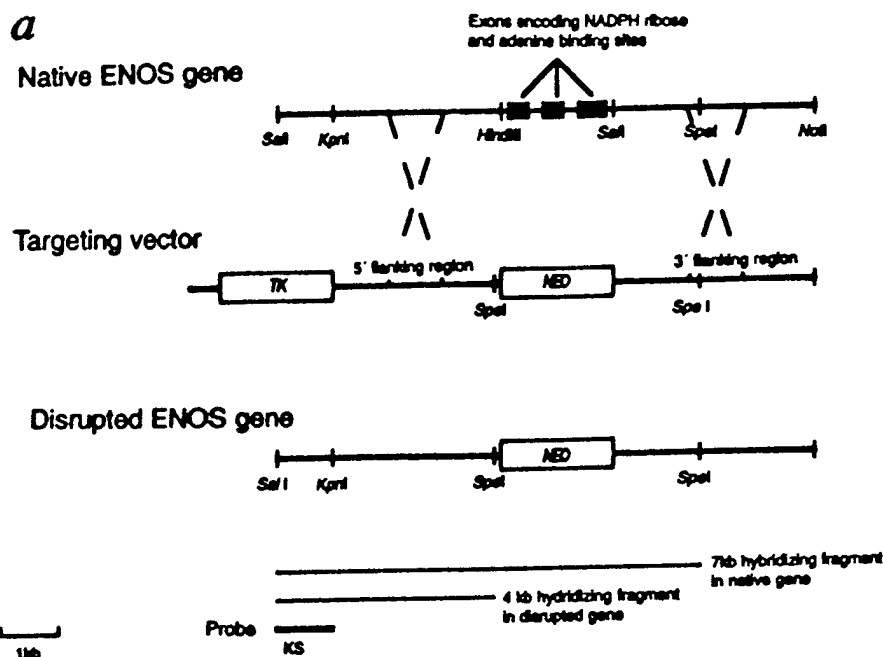
For down-regulating eNOS gene expression in the C3L5 cell line, we have been trying to delete or suppress the expression by targeted disruption and antisense technology. Initially, we attempted to knockout eNOS gene in C3L5 cells with a targeting vector (pPNT-eNOS) which contains a 5' and 3' flanking region of homology with the eNOS gene, designed to replace the exons of eNOS gene encoding NADPH ribose and adenine binding sites with a *neo* cassette in it and a *tk* cassette at the upstream. This design of the targeting construct was to allow a selection of homologous recombinants

with G418 (neomycin) and gancyclovir (17). Dual selection with neomycin and gancyclovir was designed to select for neo-resistant cells, resulting from the integration of the construct into the cellular genome, and at the same time to eliminate cells which were *tk* (Thymidine Kinase) positive. Selection against *tk* positive cells was to reduce the number of cells which would insert the construct by nonhomologous (random) means without a disruption of the eNOS gene. A further selection of cells with a higher concentration of G418 (neomycin) alone was then carried out to improve the chances of selecting cells with eNOS disruption in both the chromosomes (homozygous) rather than a single chromosome(heterozygous). The above targeting construct was kindly provided to us by Dr. Paul Huang at Harvard who disrupted the eNOS gene in embryonal stem (ES) cells to produce eNOS knockout mice (17).

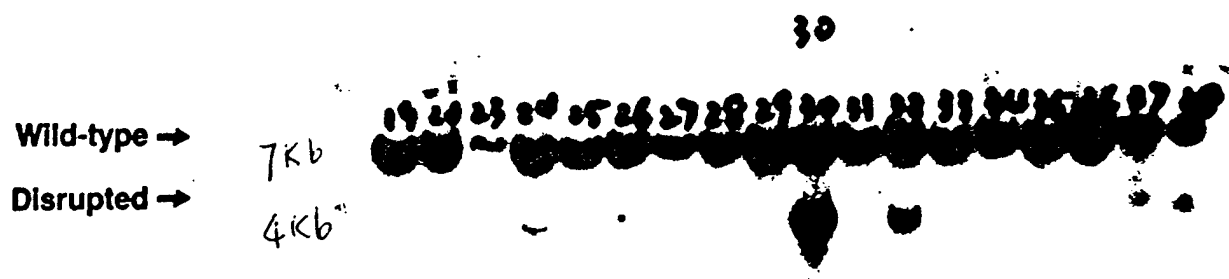
We introduced the targeting construct into C3L5 cells by two methods: either electroporation or LipofectAMINE (GIBCO) treatment. Both methods gave good results for G418-resistant colony formation. We electroporated C3L5 cells under two different conditions, 960 uF, 25 V; 250 uF, 250 V in the presence of linear pPNT-eNOS at doses of 12.5 µg, 25 µg, 50 µg, and 100 µg for each of  $10^7$  cell aliquots in 1 ml transfection buffer. The former caused more cell damage than the latter condition, but there is no significant difference in the formation of G418-resistant colonies in surviving cells. The selection of transfected cells started after 48 hours by inclusion of 400 µg/ml of G418 (GIBCO) and 2 µM of gancyclovir in the medium. Positive colonies were amplified and DNA samples were genotyped by Southern blot with 5'-probe of eNOS gene to check for wild type eNOS and disrupted eNOS, so that wild type, heterozygous and homozygous recombinants can be discriminated. Among 262 G418-resistant colonies transfected with 12.5 µg or 25 µg pPNT-eNOS, there was no heterozygous recombinant detected. In 356 G418-resistant colonies transfected with 50 µg or 100 µg of pPNT-eNOS, three colonies were identified which resembled possible heterozygous recombinants by Southern blot analysis. They were from cells transfected with 100 µg rather than 50 µg of pPNT-eNOS. These clones had DNA bands for wild type eNOS as well as a band similar to the one expected for disrupted eNOS (fig. 1). But surprisingly, in later repeats of southern analysis with these clones, we could not reproduce the data on the second band indicating that the additional band (representing disrupted eNOS) in our earlier Southern blot was an artifact, since we could not offer a biological explanation for this phenomenon. We have since continued our attempts with these 3 colonies by increasing the selection pressure with G418 at concentration of 4 mg/ml. We isolated 54 colonies, none of which revealed the genotype of either a heterozygous or homozygous recombinant.

The reason for this failure so far is unclear. We suspect that C3L5 tumor cell line may have more than two copies of the eNOS gene. To investigate this possibility one has to use fluorescent *in situ* hybridization (FISH) for the eNOS gene. We have done a karyotype analysis of the cell line showing that the cell line is hyperdiploid with a chromosome mode of 56 (figures 2 and 3). We are currently hoping to test whether there are more than two copies of the chromosome number 5 which contains the eNOS gene, by analyzing the G-banding patterns of the chromosomes. At the same time, we have tested whether wild type C3L5 cells may contain neo-resistant cells. To our satisfaction, we have been unable to produce neo-resistant colonies at high G418 concentrations tested on wild type C3L5 cells. This is why we have not given up our attempts to disrupt the eNOS gene with the procedure described above. However, we felt that we must adopt the alternate approach of antisense technology to downregulate the expression of eNOS in C3L5 cells, since the knockout experiment may not succeed.

The antisense study for suppressing eNOS gene expression in C3L5 cells has started a few months ago. Since eNOS gene has recently been cloned in the mouse by Dr. Philip Marsden (48), we have recently obtained a 2.4 kb mouse eNOS cDNA from Dr. Marsden in order to make antisense eNOS construct. Prior to antisense transfection we wish to introduce the LacZ gene into C3L5 cells in order to identify spontaneous micrometastasis of C3L5 cells in the lungs resulting from the primary subcutaneously transplanted tumors. LacZ will be introduced into C3L5 cells by infection with a retrovirus engineered with LacZ gene as well as neo-resistance gene, named BAG (beta-gal at gag) virus. LacZ positive cells will be selected with G418. LacZ will serve as a marker for micrometastatic foci by histochemical staining with X-gal. Subsequently, antisense and sense murine eNOS constructs will be introduced into these cells, and transfectants will be selected with puromycin. pPGK-puro vector plasmid (Dr. Naus, University of Western Ontario, London, ON) includes a puromycin resistance gene driven by PGK promoter, which confers the transfected cells resistance to puromycin. The ligation of pPGK-puro and eNOS cDNA fragment has been done successfully and hopefully, the antisense and sense murine eNOS plasmid can be available in the near future for the next step of transfection of C3L5 cells carrying the LacZ marker gene.



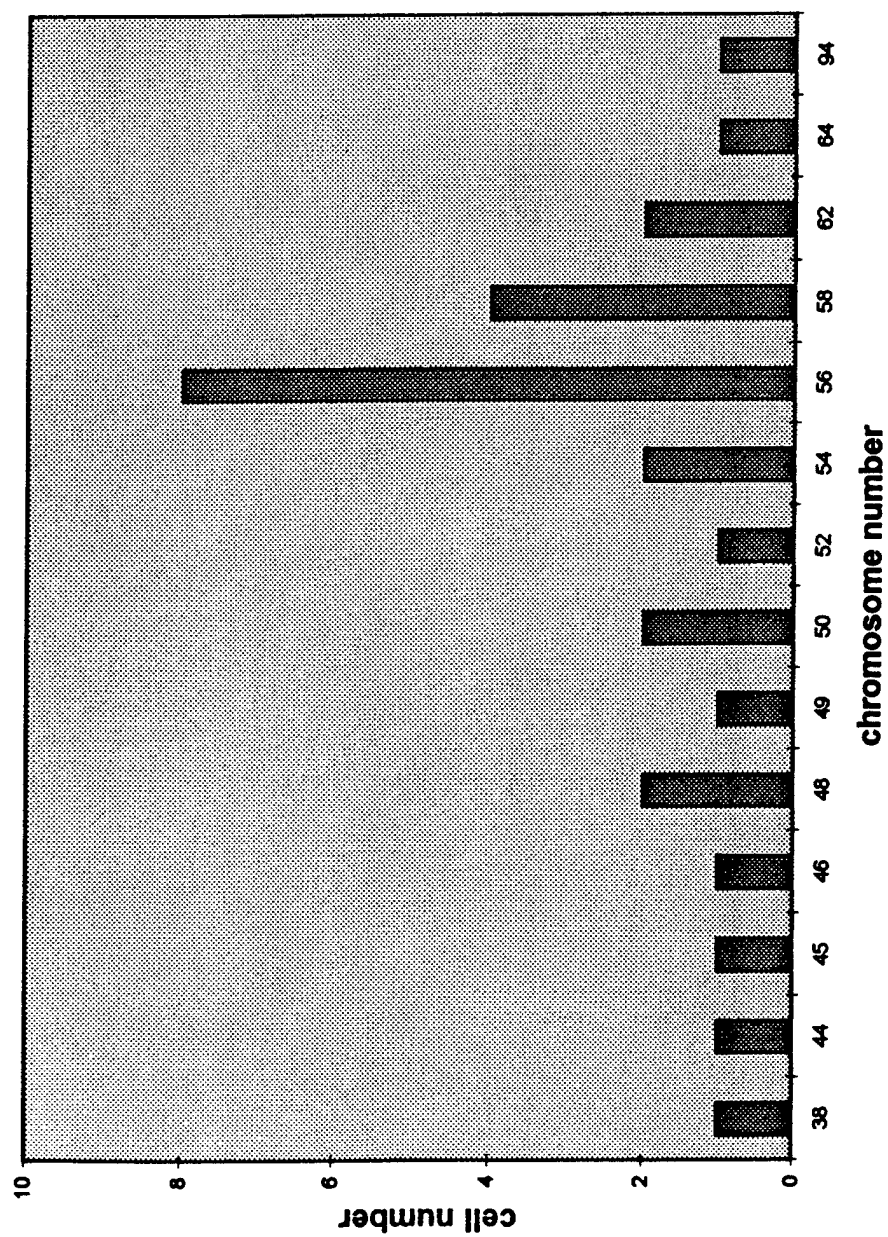
**b**



**Fig 1. Targeted disruption of the endothelial NOS gene.**

- Restriction maps of the native mouse endothelial NOS gene, the targeting vector, and the disrupted eNOS gene are shown.
- Primary results of Southern blot analysis with murine eNOS gene probe. Genomic DNA samples were isolated from G418-resistant colonies (400ug/ml), digested with SpeI. The native eNOS gene(+/+) shows a 7kb hybridizing fragment, whereas the properly targeted eNOS gene(-/-) shows a 4kb hybridizing fragment. The heterozygotes(+/-) contain both bands at 7kb and 4kb with a equal distribution in amount (see clone# 30).

Fig 2. Distribution of Chromosomes in C3-L5 Cell Line





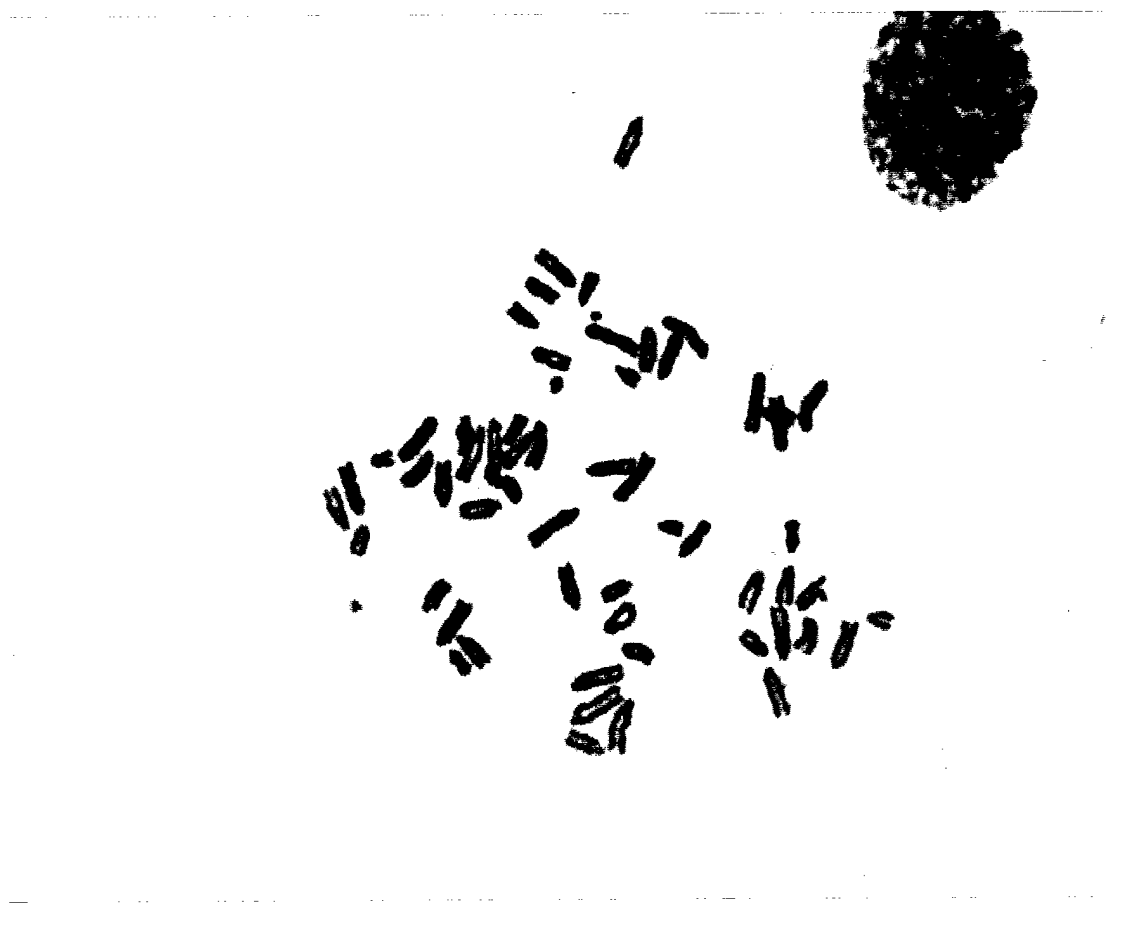


Fig 3. Karyotype of C3-L5 cells. The metaphase cells stained with Orcein, 1,000X.

**Task 2 Identification of mechanisms of tumor progression which are stimulated by NO.** We hypothesized that tumor-derived NO facilitates tumor progression and metastasis by (a) promoting tumor cell invasive ability, (b) promoting tumor cell migratory ability and (c) promoting tumor-induced angiogenesis which is critical for the growth of solid tumors. We had already shown that tumor-derived NO exerted no influence on tumor cell proliferation *in vitro*. Others have shown that tumor-derived NO promotes tumor blood flow and microcirculation which can indirectly promote tumor growth (49-51).

**(a) Effects of tumor-derived NO on the invasiveness of C3L5 tumor cells.** The results up to date have been reported in Appendix 1 (ref. 25). Here we provide a comprehensive summary.

#### **Materials and Methods:**

C3L5 tumor cells were tested for their invasive ability in an *in vitro* invasion assay (52) designed in our laboratory. In brief, invasive ability of tumor cells were measured from the ability of the cells to transgress a reconstituted basement membrane (matrigel) barrier. Tumor cells were labeled with 3HTdR for a 48 hr period in culture, washed, and placed on a matrigel-coated millipore membrane (8µm pore size) which forms the bottom of a transwell. The transwells are then placed in tissue culture wells to create an upper and a lower chamber inclusive of tissue culture medium. The degree of invasion is computed as the percent of radioactivity (β counts) appearing in the bottom chamber and the undersurface of the transwell during the assay period (24-72 hrs). The effects of adding NOS inhibitors (NMMA, L-NAME in various concentrations) with or without excess L-arginine (which competes with NOS inhibitors and abolishes their effects), or adding NOS inducers (LPS + IFN-γ) with or without NOS inhibitors were investigated. Simultaneously, the level of NO production was measured in the medium. This was done by measuring NO + NO<sub>3</sub> levels by colorimetry following reaction with Griess-reagent.

#### **Results:**

**(a)** Presence of either NOS inhibitor (NMMA or L-NAME) reduced the invasion index. This reduction was associated with a parallel reduction in the level of NO produced.

- (b) Additional presence of excess Larginine in the assay along with NOS inhibitors abrogated the anti-invasive effects of NOS inhibitors.
- (c) Addition of LPS + IFN- $\gamma$  led to strong stimulation of NO production and a simultaneous stimulation of invasiveness.
- (d) Inclusion of NOS inhibitors with LPS + IFN- $\gamma$  caused a partial abrogation of the stimulation of invasiveness and NO production by LPS + IFN- $\gamma$ . It was likely that the NO induction was too high for the inhibitors.

### **Conclusions:**

These results show that tumor-derived NO under native conditions as well as conditions of stimulation by LPS + IFN- $\gamma$  promoted the invasiveness of C3L5 cells. We are currently conducting additional experiments in order to duplicate the results.

### **(b) Mechanisms underlying the invasion promoting effects of NO.**

Invasion is a multistep process requiring attachment of cells to the constituents of the basement membrane or extracellular matrix (ECM) via integrins or non-integrin receptors, degradation of the ECM components by secretion of a variety of matrix degrading enzymes, most typically, matrix metalloproteases (MMP's) and migration of cells through the degraded matrix. Since matrix degradation is the most critical step of all, we examined whether NO-mediated stimulation of invasiveness of C3L5 cells was due to an upregulation of MMP's or a downregulation of natural MMP inhibitors - tissue inhibitors of metalloproteases (TIMP's), that is, an alteration in the balance between MMP's and TIMP's.

### **Method**

Northern analysis was carried out with total RNA extracted from C3L5 cells grown under different experimental conditions using probes for MMP-2 (72 Kda Type IV collagenase or Gelatinase A), MMP-9 (92 Kda Type IV collagenase or Gelatinase B), TIMP-1, TIMP-2 and TIMP-3. Phosphoimage analysis of Northern blots relative to 18S ribosomal RNA (loading controls) provided a measure of mRNA expression under different experimental conditions such as: control untreated cultures, cells cultured with IFN- $\gamma$  and LPS (to induce iNOS which has been shown to increase NO production by these cells) with or without NOS inhibitor NMMA, and cells cultured with NMMA alone (to block endogenous NO production via eNOS).

## Results and Conclusions:

A comprehensive summary of the results are presented in Appendix 1; and image analysis data are presented in Table 1, in which data in control cells were normalized to 1.0.

**TABLE 1. Image analysis\* of Northern blots of mRNA expression  
in C3L5 cells**

Transcript	Control	IFN- $\gamma$ and LPS	IFN- $\gamma$ and LPS + NMMA	NMMA
eNOS (4.5 kb)	1.0	1.0	1.0	1.0
iNOS (4.8 kb)	—	+	++	—
MMP-9 (2.5 kb)	—	—	—	—
MMP-2 (3.1 kb)	1.0	1.7	0.9	1.0
TIMP-1 (3.1 kb)	1.0	0.9	1.0	1.0
TIMP-2 (3.5 kb & 1.0 kb)	1.0	0.8	0.6	1.3
TIMP-3	1.0	0.3	0.6	1.2

\*Standardized with 18S RNA used as a loading control. A positive expression in control cells is normalized to 1.0.

IFN- $\gamma$  = 500 u/ml; LPS = 10  $\mu$ g/ml; NMMA = 1 mM

In brief, that data in Table 1 revealed that:

**(a)** C3L5 cells expressed eNOS but not iNOS under native conditions, however, iNOS was induced in the presence of IFN- $\gamma$  and LPS.

**(b)** NMMA treatment alone (to block eNOS, a constitutive and low level NO generator), did not alter MMP-2 expression but upregulated the expression of TIMP-2 and to a smaller level TIMP-3. This indicated that invasion stimulating effects of endogenous NO are, at least in part, mediated by downregulation TIMP-2 and possibly TIMP-3.

**(c)** IFN- $\gamma$  and LPS treatment upregulated MMP-2 and downregulated TIMP-3. This indicated that higher levels of NO induction stimulated MMP-2 production in addition to suppressing TIMP-3 production.

**(d)** IFN- $\gamma$  + LPS treatment restrained MMP-2 expression to control levels, however, only partially restored TIMP-3 expression. This explained the incomplete abrogation of IFN- $\gamma$  + LPS-induced NO production and stimulation of invasiveness when cells were treated with NOS inhibitors.

In conclusion, invasion promotion by NO in this tumor system was explained by an alteration in the balance between MMP-2 and TIMP-2/TIMP-3. An upregulation of the former occurred at high NO levels, and a downregulation of the latter occurred at all NO levels. Further studies are needed to demonstrate that the activity of MMP-2 enzyme and its inhibitors TIMP-2/TIMP-3 were altered under the same conditions, by employing zymography and reverse zymography. It would also be interesting to see whether plasminogen activation (PA) is produced by C3L5 cells and whether it is influenced by NO production; and whether its inhibitors PAI-1 and PAI-2 are also affected. This is because MMP-2 is activated by PA. Indeed, NO has been shown to upregulate urokinase type PA (uPA) in endothelial cells (53).

### **(c) Effects of tumor-derived NO on tumor-induced angiogenesis.**

We have obtained some very preliminary results which are summarized in Appendix 1.

#### **Methods:**

We devised an angiogenesis assay adapted from Kibbey et al. (54). Rehydrated matrigel (reconstituted basement membrane) which is liquid at 4°C and solidified at body temperature, was implanted subcutaneously in mice. The matrigel pellet, when using conventional matrigel, stimulates ingrowth of new blood vessels from the periphery of the implant possibly because of presence of angiogenic factors in the conventional matrigel.

We used growth factor-reduced matrigel (obtained from collaborative research) and found that it stimulated little or no angiogenesis on its own, but stimulated enormous angiogenesis when we included C3L5 tumor cells in the matrigel, and recovered the pellet at 5-10 days later. In preliminary experiments, we have varied the matrigel concentration, the matrigel volume and the tumor cell numbers to obtain optimal conditions for measurable angiogenesis (gross examination of the pellet and score of number of vessels in histological sections) in the implants placed in C3H/HeJ mice. One preliminary experiment has been conducted under the following conditions of treatment: **(a)** animals treated chronically with L-NAME via osmotic minipumps to block NO production *in vivo*; **(b)** animals treated with vehicle alone (controls); **(c)** animals receiving D-NAME (the dextro-derivative of NAME) which does not block NO production (specificity control for L-NAME).

### **Results and Conclusions:**

The preliminary results, based on morphological examination of the implants and implant histology, showed that: **(a)** matrigel alone implants had no angiogenesis whether or not animals were treated with vehicle alone or D-NAME or L-NAME; **(b)** tumor-inclusive matrigel implants showed rapid tumor cell growth and angiogenesis in vehicle-treated as well as D-NAME treated mice; **(c)** L-NAME therapy of mice receiving tumor-inclusive matrigel implants showed a significant reduction of tumor growth as well as angiogenesis. Figure 7 in Appendix 1 shows the gross morphology of implants in these preliminary experiments. These results suggest that endogenous tumor-derived NO (most likely owing to eNOS), and/or NO induced by the tumor implant in surrounding host cells promoted angiogenesis. We plan to validate these results in similar and other experiments in which the tumor cell number will be reduced in the implants. Other experiments will examine the cellular source of NO, as well as the mechanisms underlying the NO-induced angiogenesis.

**Task 3      Identification of the cellular source of NO-production responsible for IL-2 induced capillary leakage and mechanisms by which this NO-production compromises antitumor effects of IL-2 therapy.**

**(a)      Role of active inducible nitric oxide synthase expression in the pathogenesis of capillary leak syndrome.**

This work was initiated following the submission of the grant application and completed during the funding period. The study has now been published (ref. 47) and the details are provided in Appendix 3. It has also been reviewed in Appendix 2. A brief summary of the objectives, study design, results and conclusions are provided below.

We had earlier shown that therapy with the NOS inhibitor L-NAME ameliorated the manifestations of IL-2 therapy-induced capillary leakage in healthy and tumor-bearing mice (44, 45). The present study was undertaken in healthy C3H/HeJ mice subjected to one or two 4-day rounds of IL-2 therapy with or without combination of oral L-NAME therapy to (a) identify the cellular source of NOS activity and NOS protein induced by IL-2 therapy. (b) identify the nature of structural damage to the lungs of mice suffering from IL-2 induced pulmonary edema and pleural effusion, and (c) examine the effects of L-NAME therapy on the above parameters.

NOS protein was localized with immunohistochemical methodology. NOS activity was measured with Griess reaction. Capillary leakage was evaluated by measuring the water content in the lungs and the pleural cavities. Structural alterations in the lungs were assessed with light and electronmicroscopic histology.

Results revealed that IL-2 therapy induced the expression of iNOS protein in numerous tissues including the endothelium and muscles of the anterior thoracic wall as well as splenic macrophages. One round of IL-2 therapy resulted in high levels of iNOS activity in the anterior thoracic wall accompanied with pleural effusion. After two rounds of IL-2 therapy in mice which recovered from the first IL-2 round, pleural effusion was absent and NOS activity was not high in the anterior thoracic wall. IL-2 induced pulmonary edema after one round of IL-2 therapy correlated with significant rise in serum NO levels as well as structural damage to the lungs and its capillaries. Addition of L-NAME therapy eradicated NOS activity but not NOS expression. It also reduced IL-2 induced pulmonary edema and pleural effusion, restrained the rise of NO levels in the serum and pleural fluid, and restored the structural integrity of the lungs.

These results raised the following questions. Was the damage to the lungs and its capillaries due to a direct injury (structural damage and apoptosis) by NO, or injury by certain reaction product of NO? Recently, it has been reported that oxygen-free-radicals play a role in IL-2 therapy-induced capillary damage because it could be ameliorated with dimethylthiourea, a scavenger of oxygen-free-radicals (55). We hypothesise that formation of peroxynitrite, a potent endotheliotoxic molecule, due to a combination of NO with superoxide may be the strongest mediator of IL-2 induced capillary leakage. This hypothesis will be tested by immunostaining for nitrotyrosine in the lungs and lung capillaries in mice suffering from IL-2 induced pulmonary edema. Since cytotoxicity due to peroxynitrite is reported to be due to nitration of tyrosine-residues of intracellular tyrosine-kinases to form nitrotyrosine, nitrotyrosine provides a good marker for peroxynitrite-mediated cellular injury: If our hypothesis is correct, this marker should appear in IL-2 treated mice, and disappear or diminish in mice treated with IL-2 in combination with a NOS inhibitor L-NAME.

Furthermore, the cellular source of IL-2 therapy-induced NO remains to be identified in tumor-bearing mice, including the tumor site. It is likely that iNOS is induced in C3L5 tumor cells *in vivo* after IL-2 therapy.

**(b) Immune mechanisms responsible for NO-mediated compromise of antitumor effects of IL-2.**

We had earlier shown that addition of L-NAME therapy to IL-2 therapy augmented antitumor and antimetastatic effects of IL-2 therapy (44, 46). This suggested that IL-2 therapy-induced NO compromised the antitumor effects of IL-2. We hypothesized that IL-2 induced NO in the micro-environment of immune cells impeded the generation of antitumor killer cells - the LAK cells.

We tested this hypothesis following the submission of the original grant proposal, and the results have been published (56). Details of this study are provided in Appendix 4. A brief summary of the experimental design, results and conclusions are presented below.

In this study we examined the effects of L-NAME on IL-2-induced generation of antitumor cytotoxicity, *in vivo* as well as *in vitro*, in splenocytes of healthy C3H/HeJ mice as well as C3H/HeJ mice bearing transplants of C3L5 mammary adenocarcinoma. <sup>51</sup>Cr release assay was used to measure killer function of splenocytes against YAC-1 lymphoma (Natural Killer cell sensitive) and C3L5 adenocarcinoma (LAK cell sensitive) targets.

## **Results**

IL-2 treatment, *in vivo* (given as IL-2 therapy) as well as *in vitro* (when added to the splenocytes in culture), markedly stimulated splenocyte cytotoxicity against both tumor targets, but was associated with an increase in NO production measured in the serum or in the culture medium. Addition of L-NAME therapy to IL-2 therapy blocked IL-2 induced NO production *in vivo* (measured as nitrite and nitrate levels) and improved IL-2 induced antitumor killer function of splenocytes in both healthy and tumor-bearing mice as well as tumor regression. Addition of L-NAME to splenocytes *in vitro* also reduced IL-2 induced NO production in the medium and enhanced IL-2 induced cytotoxicity of splenocytes of healthy but not tumor-bearing mice. These differences between healthy and tumor-bearing mice (seen only with IL-2 treatment *in vitro* but not with IL-2 therapy) were likely due to the presence of additional cells in tumor-bearing mice which were induced to express iNOS and thus served as the additional source of NO. In future experiments, this possibility will be tested by immunocytochemical identification of iNOS bearing cells in the tumor-bearing mice including those in the spleen.



## Overall Conclusions

Following were the achievements (or failures) during the project period.

1. We have expanded and validated our preliminary data (presented in the original proposal) showing that:
  - (a) Spontaneous primary C3H/HeJ tumors show a heterogeneity in eNOS-bearing tumor cells; this expression was unrelated to tumor growth rate. However, metastatic foci resulting from each primary tumor was mostly eNOS positive.
  - (b) All C3L5 tumor cells (a highly metastatic clone of a spontaneous tumor) strongly expressed eNOS *in vitro*; a minority expressed iNOS under inductive conditions (IFN- $\gamma$  + LPS). When transplanted *in vivo*, most tumor cells at the primary site and majority at the metastatic site strongly expressed eNOS. C10 tumor cells (a poorly metastatic clone of the same spontaneous tumor) showed weak eNOS expression *in vitro*.

These findings substantiated further our hypothesis that eNOS expression provided an advantage for metastasis. We shall accrue more data in spontaneous and C3L5 tumors and conduct studies in C10 tumors *in vivo*.

2. Our year-long attempts to knockout eNOS gene in C3L5 cells have not succeeded. Our karyotypic data suggest that this may be due to multiple (more than 2) copies of the eNOS gene. Nevertheless, our efforts will continue. However, this led us to adopt the alternative approach of downregulating eNOS by antisense transfection. We have made significant progress along this line.
3. (a) We have shown that endogenous NO promoted invasiveness of C3L5 tumor cells. This was further stimulated by additional NO production by cells when treated with IFN- $\gamma$  and LPS because of the induction of iNOS in tumor cells.

This is the first evidence of NO-mediated promotion of tumor cell invasiveness.

- (b) We have partially identified the mechanisms responsible for NO-mediated stimulation of invasiveness. Endogenous and IFN- $\gamma$  + LPS-induced NO downregulated the expression of TIMP-2 and TIMP-3 genes. Induced NO further upregulated the expression of MMP-2 gene. Thus, NO-mediated promotion of invasiveness resulted from an alteration in the balance between MMP-2 and TIMP's.

This is the first demonstration of mechanisms for NO-mediated promotion of tumor cell invasiveness.

4. We have obtained preliminary data suggesting that endogenous NO promotes C3L5 tumor-induced angiogenesis.
5. We have shown that active, inducible NOS expression, leading to high NO production *in vivo* is responsible for IL-2 therapy-induced capillary leakage in healthy mice. We have identified the iNOS-expressing cells in the vicinity of the leakage (pulmonary edema, pleural effusion) and have shown that NOS inhibitors can restrain the IL-2 therapy-induced structural damage to the lungs.

Further studies are needed in tumor-bearing mice to identify iNOS expressing cells. Further studies are needed to test our hypothesis that NO-mediated capillary damage following IL-2 therapy is owing to the formation of peroxynitrite.

6. We have demonstrated that NO-mediated compromise of antitumor responses to IL-2 therapy is, at least in part, due to an impediment to optimal killer cell activation by IL-2, and that this impediment can be overcome by adding NOS inhibitors to IL-2 therapy.

This is an important advance in the field of cancer immunotherapy.

In summary, our progress matched our expectations in some areas. In other areas we had an accelerated progress, leading to some newer future experimentation within the overall objectives of the project.

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## APPENDICES

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**Role of Nitric Oxide in Tumor Progression: Lessons from Experimental Tumors**

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**Key Words:** Nitric oxide, invasion, metastasis, angiogenesis, mammary tumor

## Abstract

Nitric oxide (NO), a potent biological mediator, plays a key role in physiological as well as pathological processes, including inflammation and cancer. The role of NO in tumor biology remains incompletely understood. While a few reports indicate that the presence of NO in tumor cells or their microenvironment is detrimental to tumor cell survival and consequently their metastatic ability, a large body of clinical and experimental data suggest a promoting role of NO in tumor progression and metastasis. We suggest that tumor cells capable of very high levels of NO production die *in vivo*, and those producing or exposed to lower levels of NO, or capable of resisting NO-mediated injury undergo a clonal selection because of their survival advantage; they also utilize certain NO-mediated mechanisms for promotion of growth, invasion and metastasis. The possible mechanism(s) are: (a) a stimulatory effect on tumor cell invasiveness, (b) a promotion of tumor angiogenesis and blood flow in the tumor neovasculature, and (c) a suppression of host anti-tumor defense. In this review, we discuss these mechanisms on the basis of data derived from experimental models, in particular, a mouse mammary tumor model in which the expression of eNOS by tumor cells is positively correlated with invasive and metastatic abilities. Tumor-derived NO was shown to promote tumor cell invasiveness and angiogenesis. The invasion-stimulating effects of NO were due to an upregulation of matrix metalloproteases and a downregulation of their natural inhibitors. Treatment of tumor-bearing mice with NO-blocking agents reduced the growth and vascularity of primary tumors and their spontaneous metastases. We propose that selected NO-blocking drugs may be useful in treating certain human cancers either as single agents or as a part of combination therapies.

## **I. Introduction**

Progression of solid tumors is a multistage process involving genetic changes in tumor cells that provide selective advantages for growth, invasion, and metastasis due to tumor-derived (autocrine) or host-derived (paracrine) signals capable of promoting these events. Growth of primary as well as metastatic tumors can be facilitated by direct proliferation-stimulating events such as a perpetuation of positive growth-regulating signals, e.g., activation of certain protooncogenes which serve as receptors for proliferation-stimulating growth factors, or production of proliferation-stimulating growth factors. This can also result from a loss of negative growth-regulating signals, e.g., inactivation of certain tumor suppressor genes involved in cell cycle control or receptors for proliferation-blocking growth factors (1,2). Tumor growth can also be facilitated indirectly by promotion of tumor angiogenesis and tumor blood flow (3).

For tumor cells to invade into surrounding normal tissues or metastasize to a distant site, a number of steps must be completed successfully (4). First, tumor cells must bind to one or more constituents of the basement membrane or extracellular matrix (ECM) via cell surface integrins or non-integrin receptors. This binding is more than an adhesive event; it can also lead to transduction of signals that may facilitate invasion (5). Second, tumor cells must degrade basement membrane and ECM constituents; this step is facilitated by the production of active matrix degrading enzymes in excess of natural inhibitors of these enzymes (4,6). Third, tumor cells must migrate through the degraded ECM. This step is facilitated by the anchoring of cells to the ECM by appropriate integrin(s) (7), and by migration-promoting receptor-ligand interactions (8). Finally, for metastasis to occur, tumor cells must intravasate and survive within the blood vessels or lymphatics, and then

extravasate and seed at distant locations. Recent work utilizing live videomicroscopy has demonstrated that even after successful extravasation and seeding, many tumor cells may either die or remain quiescent for a significant period (9). Thus, successful metastasis often requires additional autocrine/paracrine growth or angiogenesis-stimulating signals at the new site. Similar steps when successfully repeated, may allow metastatic tumors to remetastasize to newer sites.

In the present article we shall discuss the contributory role(s) of nitric oxide (NO) in tumor progression and metastasis in the context of the above events which can influence growth, invasion or metastasis in experimental tumor models.

During the last decade, following the discovery (10) that NO accounts for the full biological activity of endothelium-derived relaxing factor (EDRF) (11), NO has been shown to be produced by many mammalian cells and responsible for numerous physiological functions. These include vasodilation, inhibition of platelet aggregation, modulation of neurotransmission, and mediation of injury by macrophages to bacteria, parasites and tumor cells (12-17). On the other hand, sustained high levels of NO production in the body can also lead to pathological injuries mediated by NO or its metabolites (18). NO production depends on conversion of the amino acid L-arginine to L-citrulline by a family of enzymes named NO synthases (NOS) (19-20). Three isoforms have been identified so far. The endothelial type NOS (eNOS) is a constitutive,  $\text{Ca}^{++}$  and calmodulin-dependent form of the enzyme, expressed by many cells including endothelial cells, myocardial cells and pyramidal cells of the hippocampus. The neuronal type NOS (nNOS) is also constitutive,  $\text{Ca}^{++}$  and calmodulin-dependent, and is expressed by certain cells including neurons of the central nervous system, the myenteric plexus, skeletal muscle cells, renal, bronchial and pancreatic islet cells. Inducible type NOS (iNOS) is  $\text{Ca}^{++}$  and calmodulin-

independent, and is expressed by macrophages in many mammalian species, endothelial cells, hepatocytes, cardiac myocytes, chondrocytes and many other cells following stimulation by inflammatory cytokines and/or bacterial endotoxin (20-23). The expression of iNOS is high in activated rodent macrophages and endothelial cells, and poor in human macrophages (24).

NO is often an important component of the chemical microenvironment of tumors, produced either by tumor cells, endothelial cells in the tumor microvasculature or macrophages and stromal cells within the tumors. Because of its lipophilic nature, NO can rapidly cross cell membranes and enter intracellular compartments to exert its action, even when produced by a neighbouring cell. Thus, it can mediate interactions between tumor cells and host cells. The functional role of NO in tumor biology is complex and remains to be fully defined. While a small number of reports indicate that the presence of NO in tumor cells or in their microenvironment is detrimental to tumor cell survival and consequently their metastatic ability, numerous clinical and experimental studies suggest a promoting role of NO in tumor progression and metastasis. In this review, we discuss these two apparently conflicting views and suggest that the opposing effects of NO may depend on two important variables: the levels of NO production and the genetic makeup of tumor cells. We suggest that in a heterogeneous population of tumor cells, clonal evolution favors those capable of resisting NO-mediated injury. In addition, many tumor cells may also utilize NO in their microenvironment, facilitating some of the steps required for tumor growth, invasion and metastasis; this will be illustrated from our own studies using spontaneous C3H/HeJ mouse mammary adenocarcinomas and their clonal derivatives as an experimental model for tumor progression and metastasis.

## **II. Association between NO and tumor growth**

The genotoxic role of NO in promoting carcinogenesis is well recognized. The underlying mechanisms are the subject matter of another article (by Felley-Bosco) in this issue. Chronic exposure of cells to NO can result in multiple genetic changes which may underlie histological changes such as metaplasia and the progression of metaplasia into neoplasia. The presence of eNOS in breast apocrine metaplastic cells of fibrocystic disease in the human (25) and iNOS in macrophages within the hyperplastic stromal tissue of a rat model of Barret's esophagus (26) are believed to promote the progression of metaplastic epithelia into carcinomas.

Human tumor materials, in general, have provided the strongest link between NO production and disease progression. While the functional implications of elevated serum NO levels observed in many cancer patients (27) remain unexplored, a number of reports indicate a contributory role of NO to tumor progression. An abundant expression of NOS, as well as NOS activity, has been positively correlated with the degree of malignancy in human ovarian and uterine cancers (28), central nervous system tumors (29), and breast cancer (30). Contributing to the elevated NOS activity are constitutive form(s) in tumor cells (28,29) and/or tumor endothelial cells (29), and the inducible form in the tumor endothelial cells (29) and/or tumor associated macrophages (30).

The relationship of NO to human colonic tumor progression remains controversial. Histochemical localization of NAD(P)H diaphorase enzyme, NOS activity and NOS expression in the human colonic mucosa, polyps and carcinomas suggest an inverse relationship between the enzymes and colonic tumor progression (31,32). In contrast, studies of NOS gene expression and NOS activity in a panel of human colonic adenocarcinoma cell lines revealed that all expressed mRNA for the eNOS gene, and some exhibited significant NOS activity (33). Evidently, more studies are needed in human colonic tumors.

NOS expression has been examined in a number of experimental tumor models. An abundant expression of iNOS by cells of the tumor vasculature has been implicated in the promotion of tumor growth both in murine (34) and rat (35) tumors. eNOS expression by tumor cells is positively correlated with invasiveness and metastasis in a murine mammary adenocarcinoma model, to be described later.

Numerous studies in animal models have provided direct evidence for a stimulatory role of NO in tumor progression. In a rat colonic adenocarcinoma model showing iNOS expression in the tumor vasculature, treatment with N<sup>G</sup>-Nitro-L-arginine methyl ester (L-NAME), a potent NOS inhibitor, reduced NO production and tumor growth (35). Similarly, anti-tumor and anti-metastatic effects of two NOS inhibitors N<sup>G</sup>-methyl-L-arginine (NMMA) and L-NAME were observed in our laboratory using a mouse mammary adenocarcinoma model (36,37), in which tumor cells expressed eNOS. Recently, Edwards *et al.* (38) observed that NO production induced by lipopolysaccharide (LPS) and interferon (IFN)- $\gamma$  in EMT-6 murine breast cancer cells inhibited cell growth *in vitro*, but stimulated tumorigenesis and experimental lung metastasis *in vivo*. Finally, engineered expression of murine iNOS in a human colonic adenocarcinoma cell line resulting in continuous, moderate levels of NO production *in vitro*, was associated with increased tumor growth and vascularity *in vivo* following transplantation in nude mice (39). These findings of a facilitatory role of NO in tumor growth and metastasis are in contrast with those reported for murine K1735 melanoma cell lines, in which the level of iNOS expression was inversely correlated with their ability for experimental metastasis (40). Furthermore, engineered overexpression of iNOS in an iNOS-deficient melanoma line suppressed tumorigenic and metastatic abilities *in vivo* because of NO-mediated cytostasis and apoptosis (41). Two explanations may be offered for these apparently conflicting results. First, very high NO levels (such as those produced by the iNOS overexpressing murine melanoma line) can be detrimental to tumor cell survival. Indeed, the iNOS overexpressing melanoma line had poor survival in the absence of NOS

inhibitors *in vitro* and *in vivo* (41). Second, tumor cells may vary in their susceptibility to NO-mediated cytostasis and apoptosis; certain tumor cells can not only resist NO-mediated injury, but also utilize NO to facilitate tumor progression and metastasis. These possibilities will be discussed later in more detail.

### **III. C3H/HeJ spontaneous mammary adenocarcinoma and its clonal derivatives: a model for tumor progression**

Approximately 90% of C3H/HeJ female retired breeder mice develop mammary tumors during their lifespan (42). Despite extensive variation in the site (anywhere in the mammary line) or the time (6 months to 2 years) of tumor appearance, most tumors are highly vascular and exhibit histological features of invasive adenocarcinomas with a pseudoglandular architecture. In general, animals demonstrating spontaneous primary tumors develop eventual metastasis in their lungs. Lung metastasis may occasionally be present even in the absence of a visible or palpable primary tumor (Lala, Al Mutter and Orucevic, submitted for publication). Tumor development in these mice requires the proviral form of the mouse mammary tumor virus (MMTV), which is transmitted via the mother's milk and integrated in the developing mammary tissue of the female offspring. This leads to tumorigenesis owing to insertional mutagenesis of certain important growth-regulating genetic loci which serve as the proviral integration sites (43,44).

Clonal derivatives of spontaneous mammary tumors exhibited extensive heterogeneity in growth rates observed *in vitro* and *in vivo*, and metastasis formation *in vivo* following subcutaneous transplantation in syngenic mice (45). Two clones derived from a single spontaneous tumor differed markedly in their abilities for spontaneous lung metastasis from a primary subcutaneous transplant site; C10 was poorly metastatic, and C3 was highly metastatic. However, because the metastatic ability of C3 declined after several years of *in vitro* passage (46), C3 cells were subjected to an *in vivo* selection pressure of 5 cycles of subcutaneous to pulmonary passage, yielding highly metastatic



cells (47). The resulting cell line, designated C3L5, has since maintained a very high ability for spontaneous lung metastasis from subcutaneous sites. The data presented below relate to C3H/HeJ spontaneous mammary tumors as well as the high and low metastatic clonal derivatives, C3L5 and C10, respectively.

#### **IV. Relationship of NO production to tumor growth and metastasis in the C3H/HeJ mammary tumor model**

##### **IV. A. NOS expression in spontaneous (primary and metastatic) mammary tumors (Lala, Orucevic and Hum, unpublished)**

Random examination of spontaneously developing mammary tumors harvested at 12 weeks of tumor age revealed immunohistochemical evidence of eNOS expression in tumor cells (Fig. 1A and B) and iNOS expression in certain macrophages in the tumor stroma (Fig. 1C). Endothelial cells in the tumor vasculature were eNOS positive.

In the primary tumors, a heterogenous pattern of eNOS staining was noted; tumor cells in pseudoacinar formation were either strongly positive or negative (Fig. 1A). However, the lung metastatic nodules were composed primarily of strongly eNOS positive cells (Fig. 1B), suggesting a positive correlation between eNOS expression and metastatic ability.

##### **IV. B. NOS expression in C3L5 and C10 tumor lines (Lala, Orucevic and Hum, unpublished)**

C3L5 and C10 tumor lines, when transplanted subcutaneously, both give rise to primary tumors which grow to large sizes. However, C10 tumors grow more slowly, are more circumscribed and less invasive. The mean number of spontaneous lung metastases produced 3 weeks after subcutaneous transplantation of  $5 \times 10^5$  cells in syngeneic mice ( $n = 15$ ) was 10.7 (median = 8) with C3L5 cells and 1.3 (median = 1) for C3L5 and C10 cells, respectively.

*In vitro* cultured tumor lines showed positive eNOS staining in nearly 100% of C3L5 cells (Fig. 2A), but weak (and heterogeneous) eNOS staining in C10 cells (data not shown). Both cell lines were negative for iNOS. When cultured in the presence of LPS and IFN- $\gamma$ , C3L5 cells were induced to express iNOS as shown by strong iNOS staining in 25-30% of cells (Fig. 2B). C3L5 cells grown *in vivo* maintained strong positivity for eNOS, both in the primary tumors and their spontaneous lung metastases (Fig. 1C and D). These findings were consistent with the notion that eNOS expression by tumor cells provided a selective advantage for invasion and metastasis in this mammary tumor model.

#### **IV. C. Effects of NOS inhibitors (NMMA and L-NAME) on tumor growth and metastasis in C3L5 tumor-bearing mice**

Treating C3L5 tumor-bearing mice with two NOS inhibitors (36,37) provided the first direct evidence of a contributory role of NO in tumor growth and metastasis. NMMA (given repeatedly as two 3 day rounds by the subcutaneous route) as well as L-NAME (administered orally in the drinking water as two 4 day rounds) led to a reduction in the growth of the subcutaneously transplanted primary tumors and their spontaneous lung metastases (Figs. 3 - 6). The finding of reduced primary tumor growth has since been reproduced with chronic L-NAME therapy administered subcutaneously using osmotic minipumps (Jadeski and Lala, unpublished). Treatment with L-NAME has also been reported to reduce the growth of the primary tumors in a transplanted rat colonic adenocarcinoma model (35), in which the tumor vasculature expressed iNOS. Based on the temporal kinetics of tumor growth after the therapy, the authors suggested that L-NAME reduced blood flow through the tumor vasculature and that native NO was instrumental in promoting the tumor blood flow.

What are the mechanisms underlying NO-mediated promotion of tumor growth and metastasis observed in numerous tumor models? The possibilities, in theory, include: (a) a direct stimulation of tumor cell proliferation; (b) a promotion of tumor cell

invasiveness; (c) a promotion of tumor angiogenesis; (d) a promotion of microcirculation in the tumor neovasculature; (e) a suppression of the host anti-tumor defence. Of these, we have gathered evidence in favour of possibilities (b) and (c). The influence of NO in tumor microcirculation is the subject matter of another article (by Fukumura and Jain) in this issue.

#### **V. NO and tumor cell proliferation, survival and apoptosis**

Edwards *et al.* (38) found a discrepancy in tumor cell proliferation *in vitro* and tumor growth *in vivo* after induction of NO with LPS and IFN- $\gamma$ ; *in vitro* induction inhibited tumor cell proliferation, whereas *in vivo* induction promoted tumorigenesis and metastasis. Since C3L5 mammary adenocarcinoma cells expressed active eNOS and produced NO *in vitro*, we tested whether NMMA affected tumor cell proliferation by measuring the uptake of  $^3\text{H}$ -Thymidine ( $^3\text{HTdR}$ ) by C3-L5 cells *in vitro*. NMMA treatment for 24 hours at concentrations capable of blocking NO production *in vitro*, had no influence on  $^3\text{HTdR}$  uptake when cells were pulsed with  $^3\text{HTdR}$  during the last 6 hours of 24 hour culture (unpublished data), suggesting that endogenous NO did not directly affect C3L5 tumor cell proliferation.

It has been reported that endogenous or exogenous NO exerts anti-proliferative effects on cells that express functional wild-type p53 tumor suppressor gene (48,49). NO stimulates accumulation of the p53 protein in these cells which blocks proliferation by hindering progression of cells through the cell cycle; this may explain cytostatic effects of NO on certain tumor cells. Tumor cells transfected with iNOS were growth-inhibited *in vivo* only when they expressed wild type p53. However, iNOS-expressing tumor cells in which p53 was lost or mutated were resistant to the anti-proliferative effects of endogenous NO, and grew faster *in vivo* than those not expressing iNOS (49).

Apoptosis is another mechanism which can compromise cell survival in the presence of high NO levels. It has been suggested that the NO-dependent component of tumoricidal function of certain immune effector cells is by induction of tumor cell apoptosis (50). A similar tumoricidal function has also been ascribed to cytokine-activated endothelial cells (51). Very high levels of endogenous NO production can trigger apoptosis in the NO-producing cell, e.g. in cytokine-activated transformed murine fibroblasts (52). This phenotype can be detrimental to tumor cell survival. For example, engineered iNOS overexpression in a murine melanoma line abrogated tumorigenic and metastatic ability of these cells because of rapid apoptosis; these cells failed to survive even *in vitro* in the absence of NO-blocking agents (41). However, tumor cells can vary widely in their susceptibility to NO-mediated apoptosis, from being highly susceptible to totally resistant. Reasons for such variation have not been fully explored. A variation in the genetic makeup can be suggested as one of the reasons. For example, susceptibility is provided by the expression of wild-type p53, and cellular ability to upregulate functional p53 in response to NO, whereas loss or mutation of p53 makes the cells resistant to NO-mediated apoptosis (49, 53,54). Furthermore, an overexpression of Bcl2 can protect tumor cells from NO-mediated apoptosis (55,56).

## **VI. NO and Tumor Cell Invasiveness**

### **Role of tumor-derived NO in the invasiveness of C3L5 cells**

This was tested in an *in vitro* matrigel invasion assay (57), in which the invasive ability of tumor cells was determined by measuring the proportion of tumor cells transgressing a matrigel barrier during a 24-72 hour period, after blocking endogenous NO production or inducing additional NO production. Specifically, the effects of adding NOS inhibitors (NMMA, L-NAME in various concentrations) with or without excess L-arginine (which competes with the NOS inhibitors, abolishing their effects), or adding iNOS inducers (LPS in combination with IFN- $\gamma$ ) with or without NOS inhibitors were

examined. The results can be summarized as follows. (i) Presence of NMMA or L-NAME reduced the invasion index; a parallel reduction in the level of NO production by the tumor cells measured by the  $\text{NO}_2^- + \text{NO}_3^-$  levels in the medium was observed. (ii) Inclusion of excess L-arginine with NOS inhibitors abrogated the anti-invasive effects of the NOS inhibitors, attesting to the functional NOS specificity of the inhibitors. (iii) Inclusion of LPS and IFN- $\gamma$  led to a major stimulation of NO production and a concomitant stimulation of invasiveness. (iv) Inclusion of NOS inhibitors along with LPS and IFN- $\gamma$  caused only a partial reduction in invasiveness, and LPS and IFN- $\gamma$ -induced NO production. These findings clearly demonstrated that constitutive NO production by C3L5 tumor cells upregulated their invasive ability. The invasion-stimulating effects of LPS and IFN- $\gamma$  could be partially explained by a stimulation of NO production.

What are the mechanisms underlying the invasion-stimulating effects of NO in this tumor model? Earlier studies have shown that NO promotes degradation of articular cartilages by activating matrix metalloproteases (MMP's) in chondrocytes from numerous species (58,59). We hypothesized that NO leads to an alteration in the balance between the synthesis of MMP's and the synthesis of their natural inhibitors, i.e., tissue inhibitors of metalloproteases (TIMP's) in tumor cells. To test this hypothesis, the levels of MMP and TIMP mRNA expression were measured in these cells under different experimental conditions. C3L5 cells expressed the 72 kDa type IV collagenase (gelatinase A) and not the 92 kDa species (gelatinase B). These cells also expressed TIMP-1, TIMP-2 and TIMP-3. Phosphoimage analysis of Northern blots relative to the 18S RNA (loading controls) provided a measure of the mRNA expression under the various experimental conditions. The results are presented in Table 1.

Data presented in Table 1 show that (i) C3L5 cells expressed eNOS but not iNOS mRNA under native conditions, however, iNOS expression was induced in the presence of LPS and IFN- $\gamma$ . This induction was upregulated by treatment of cells with NMMA, explained by a reduction of the NO-mediated negative feedback on the iNOS gene

expression. (ii) NMMA treatment did not affect MMP-2 expression, but upregulated the expression of TIMP-2, and to a minor extent TIMP-3, indicating that the invasion-stimulating effects of endogenous NO are, at least in part, mediated by a downregulation of TIMP-2, and possibly TIMP-3. (iii) LPS and IFN- $\gamma$  treatment upregulated MMP-2 and down-regulated TIMP-3, explaining the invasion-stimulating effects. Addition of NMMA to LPS and IFN- $\gamma$  restrained the MMP-2 expression to normal level and only partially restored TIMP-3 expression, thus explaining the incomplete abrogation of LPS and IFN- $\gamma$  stimulation of invasiveness with NOS inhibitors. These results indicated that LPS and IFN- $\gamma$ -mediated stimulation of invasiveness is only partially explained by increased NO production, and that NO at higher levels can upregulate MMP-2 and downregulate TIMP-3. Indeed, we have observed that exposure of C3L5 cells to S-Nitroso-N-Acetyl-D, L-penicillamine (SNAP) (an NO donor) downregulates TIMP-3 mRNA (data not shown). Assuming that gene expression was directly related to the secretion of protein products, these results suggest that NO-mediated stimulation of invasiveness is due to an alteration in the balance between productions of the MMP's and TIMP's. Furthermore, NO has been shown to upregulate urokinase type plasminogen activator (uPA) in endothelial cells of post capillary venules during the process of NO-mediated stimulation of angiogenesis (60). Since uPA converts plasminogen to plasmin, which can activate numerous MMP's, this may represent another pathway of NO-mediated stimulation of matrix degradation.

Further studies of the relationship of NO to tumor cell invasiveness are needed using different tumor models. In K1735 murine melanoma cells, engineered overexpression of iNOS, leading to decreased tumor cell survival and tumorigenicity (41), has been reported to be associated with a down regulation of MMP-2 (61) owing to a downregulation of MMP-2 promoter activity. It is thus possible that very high levels of endogenous NO may compromise invasive function of certain tumor cells, which are susceptible to NO-mediated cytotoxicity.

## **VII NO and Angiogenesis**

### **VII A. Roles of NO In angiogenesis under physiological conditions and during wound healing**

Regulatory roles of NO in angiogenesis remain somewhat controversial. Pipili-Synetos *et al.* (62) suggested that NO is an endogenous inhibitor of angiogenesis. Using an angiogenesis assay which scores the number of blood vessels in a defined area of chick chorio-allantoic membrane (CAM assay), as well as tube formation by endothelial cells on matrigel, they found that Na-nitroprusside (an NO-donor) reduced and NOS inhibitors promoted basal angiogenesis. In contrast, Konturek *et al.* (63) found that inhibition of NO synthesis delayed healing of chronic gastric ulcers induced by acetic acid, by reducing local blood flow and angiogenesis at the periphery of the ulcers. Similarly, using both an *in vivo* angiogenesis assay with rabbit cornea and an *in vitro* assay which measures the growth and migration of capillary endothelial cells, Ziche *et al.* (64) showed that vasoactive substances such as substance P, or prostaglandin E (PGE)<sub>1</sub> stimulated angiogenesis in an NO-dependent manner, since it was blocked with NOS inhibitors NMMA, N<sup>G</sup>-nitro-L-arginine (L-NNA) and L-NAME. NO donors such as Na-nitroprusside and glycerol trinitrate also stimulated endothelial cell migration. These authors have reported that one of the final pathways of NO-mediated angiogenesis is by upregulation of basic fibroblast derived growth factor (bFGF) in post capillary venule endothelial cells (60). Angiogenic activity of human monocytes (65) and mitogenic activity of VEGF on coronary venular endothelium (66) have also been shown to be NO dependent.

It is possible that the conflicting data cited above on the role of NO in angiogenesis is due to the differences in levels of NO and NO scavengers in the microenvironment. Very high levels of NO may be cytostatic or apoptosis-inducing for endothelial cells, whereas low to moderate levels of NO may promote endothelial cell migration, invasiveness and differentiation, either directly or by induction of angiogenic factors such as bFGF. A continuous remodelling of blood vessels, requiring highly localized

endothelial cell death may be facilitated by high local endogenous NO levels (hot spots) in an embryonic tissue such as the CAM, which was utilized in the angiogenesis assay by Pipili-Syntos *et al.* (62). This may account for the anti-angiogenic role of NO observed using this model, as opposed to the angiogenic role of NO in other models in which the basal level of NO may be low or negligible.

#### **VII B. Role of NO in tumor angiogenesis**

Buttery *et al.* (34) suggested that iNOS expression by endothelial cells of the neovasculature of many experimental tumors promoted angiogenesis as well as blood flow in the vasculature, and thus sustained tumor growth. This suggestion has been reinforced by other investigators (67), and validated by Jenkins *et al.* (39) showing that the increased *in vivo* growth resulting from iNOS transfection of a human colon cancer cell line was associated with increased vascularity of the transplants in nude mice. The latter findings have since been confirmed with other tumor cell lines transfected with iNOS, when the tumor cell lines had lost or mutated p53 (49).

#### **Angiogenic role of NO in the C3L5 mammary tumor model (Jadeski, Hum, Orucevic and Lala, unpublished)**

We gathered two types of evidence for the angiogenic role of NO in the C3L5 tumor model. (i) Tumors of the same age in mice subjected to NMMA therapy were compared with those in animals treated with vehicle alone for the incidence of blood vessels per unit area of the section of tumor tissue (Orucevic and Lala, unpublished). Tumors in NMMA-treated mice exhibited a significant reduction in the incidence of blood vessels. (ii) We designed a tumor angiogenesis assay by adapting the protocol of Kibbey *et al.* (68). Rehydrated matrigel, which is liquid at 4°C and solidifies at body temperature, was implanted subcutaneously in mice. The matrigel pellet stimulates the ingrowth of new blood vessels from the periphery of the implant, possibly because of the presence of



angiogenic factors in conventional matrigel used by Kibbey *et al.*, (68). In our experiments, we replaced the conventional matrigel with growth factor-reduced matrigel which, on its own, stimulated little or no angiogenesis (Fig. 7A) in C3H/HeJ female mice.

However, inclusion of an appropriate number of C3L5 tumor cells in the matrigel was highly angiogenic. When animals were placed on chronic subcutaneous L-NAME therapy via osmotic minipumps shortly following the implantation, both the vascularity and the size of the tumor cell-inclusive implants declined (gross appearance shown in Fig. 7C) as compared to the implants in control mice receiving vehicle alone or D-NAME therapy (Fig. 7B). None of the therapies had any influence on the vascularity of the tumor cell-exclusive implants. These preliminary data suggest that an inhibition of NO production led to a reduced angiogenic ability of the tumor cells in the implants. Whether the decline in the growth of tumor cells was secondary to the angioreductive effects alone remains undetermined.

### **VIII NO and host immune responses**

A number of reports suggest opposing roles of NO in tumor immunity. Activated murine macrophages synthesize NO (69), which may partly mediate their cytotoxic activity against tumor cells (17,50,59), bacteria (16) and parasites (71). Mills *et al.* (72) reported that ascites tumor growth in the mouse peritoneal cavity was associated with a reduced NO production by intratumor macrophages. Similarly, it has been reported that *in vitro* tumoricidal function of activated natural killer (NK) cells depends partly on their NO synthesizing ability (73-75). In contrast, NO overproduction by rodent macrophages has been shown to suppress proliferation of T lymphocytes in response to antigens or mitogens (76,77), and thus may hinder anti-tumor immune responses of T cells. Indeed, excessive NO production has been implicated in tumor-induced immunosuppression in rats (78). We tested whether a potentiation of interleukin-2 (IL-2)-induced regression of C3L5 mammary tumors (37) resulting from L-NAME therapy can be explained, at least in part,

by a potentiation of lymphokine activated killer (LAK) cell activation. We found that L-NAME treatment *in vivo* as well as *in vitro* markedly stimulated IL-2-induced generation of anti-tumor cytotoxicity of splenocytes in healthy as well as mammary adenocarcinoma-bearing mice; there was a parallel drop in IL-2-induced NO production *in vivo* and *in vitro* (79). These results revealed that the IL-2-induced increase in NO production had a compromising effect on optimal LAK cell activation, which can be overcome by NO inhibition. In our hands, NO inhibitors added during the cytotoxicity assays had no detrimental effect on LAK cell mediated anti-tumor cytotoxicity. In summary, NO appears to be an important bioactive component of the cytotoxic pathways of anti-tumor effector cells, in particular macrophages. However, sustained NO release in the immune microenvironment is also detrimental to effector cell activation pathways, and thus suppresses their anti-tumor function.

## **IX Conclusions and Suggestions**

The above evidence suggest that NO may play opposing roles in tumor growth and metastasis. The precise role of NO produced by tumor cells or host cells in the tumor microenvironment may depend on two variables: (a) the level of NO production and (b) the genetic make up of the tumor cells. Very high levels of NO can be detrimental to the survival of certain tumor cells as well as host cells because of NO-mediated cellular injury, cytostasis and apoptosis. Since high NO-producing tumor cell clones would likely delete themselves *in vivo*, it is reasonable to postulate that in most well established spontaneous tumors exhibiting a clonal heterogeneity, those producing low to moderate levels of NO or those capable of resisting NO-mediated cytostasis and apoptosis will survive and propagate. For these cells, tumor or host-derived NO may have a facilitating role for tumor progression by virtue of NO-mediated stimulation of invasiveness, angiogenesis and microcirculation within the tumor. These events may assume greater significance within the hypoxic regions of a tumor. In a solid tumor, hypoxia is known to

provide a stimulus for angiogenesis by various pathways (80). One of the mechanisms is the induction of vascular endothelial growth factor (VEGF) which has a hypoxia-responsive element in the promoter region of the gene. Hypoxia can also induce iNOS in a similar manner by activating the iNOS promoter via a hypoxia-responsive element (81). The hypoxia-induced NO production may provide additional angiogenic and invasion-stimulating signals within a solid tumor.

The genetic make up of tumor cells that may dictate susceptibility or resistance to NO-mediated injury remains to be completely investigated. One of the possible genetic determinants is the functional status of the p53 tumor suppressor gene. It has been shown that iNOS-transfected tumor cells expressing wild type functional p53 are vulnerable to NO-mediated cytostasis, because of an accumulation of p53 protein induced by endogenous NO (48,49). However, p53 accumulation eventually leads to a transcriptional transrepression of iNOS and thus improves cellular survival (48). Tumor cells in which p53 gene is deleted or mutated (causing a loss of normal p53 function), on the other hand, can withstand NO-mediated cytostasis or apoptosis (49,53,54). In addition, in the presence of endogenous NOS, p53-deficient or mutant tumor cells exhibit faster tumor growth and vascularity, when transplanted *in vivo* (49). These observations led to the hypothesis that interaction of NO with p53 provides one mechanism of clonal selection of p53 mutant or p53 null cells which can utilize NO to their advantage for tumor progression *in vivo* (49,82). Since p53 mutation occurs in nearly half of all human cancers (83), this hypothesis predicts that NO would facilitate progression in a large proportion of human cancers. It remains to be seen whether a p53 dependent role is universal to all tumors and whether other tumor suppressor genes, e.g., Rb may interact with NO in a similar manner. The picture can be complicated further by other (nongenetic) mechanisms of protection from NO-mediated injury. For example, certain cells show acquired resistance to NO-mediated injury after prior exposure to NO (84,85). Nevertheless, availability of reliable genetic markers which can predict the specific role of NO in tumor biology will be

highly valuable in determining the applicability of NOS inhibitors in treating specific tumors.

Table 2 presents a schema of the variables that may dictate the role of NO in tumor biology.

#### **X Key unanswered questions**

1. Can the opposing roles of NO (on tumor progression vs. tumor regression) be explained by (a) the level of NO production, eg. high vs. moderate to low, (b) the ability of the tumor cell type to resist NO-mediated injury, or (c) both? Further experimentation using different tumor systems is needed to answer these questions. In particular, genetic determinants which may allow tumor cells to resist NO-mediated injury and exploit NO to their advantage deserve full exploration.
2. How universal is the phenomenon of NO-mediated promotion of tumor angiogenesis? This area needs further investigation using multiple tumor models with different NO producing abilities *in vivo*.
3. What are the effects of disrupting or down-regulating the eNOS gene in the high eNOS-expressing and highly metastatic C3L5 mammary tumor cells on their invasive, angiogenic and metastatic abilities? Conversely, what are the results of upregulating eNOS in the low eNOS expressing, poorly metastatic C10 mammary tumor cells?

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**TABLE 1. Image analysis\* of Northern blots of mRNA expression  
in C3L5 cells**

Transcript	Control	IFN- $\gamma$ and LPS	IFN- $\gamma$ and LPS + NMMA	NMMA
eNOS (4.5 kb)	1.0	1.0	1.0	1.0
iNOS (4.8 kb)	—	+	++	—
MMP-9 (2.5 kb)	—	—	—	—
MMP-2 (3.1 kb)	1.0	1.7	0.9	1.0
TIMP-1 (3.1 kb)	1.0	0.9	1.0	1.0
TIMP-2 (3.5 kb & 1.0 kb)	1.0	0.8	0.6	1.3
TIMP-3	1.0	0.3	0.6	1.2

\*Standardized with 18S RNA used as a loading control. A positive expression  
in control cells is normalized to 1.0.

IFN- $\gamma$  = 500 u/ml; LPS = 10  $\mu$ g/ml; NMMA = 1 mM

**TABLE 2. NO and tumor biology: variables**

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**Source of NO in the tumor:**

Tumor cells (eNOS, nNOS, iNOS)

Tumor endothelium (eNOS, iNOS)

Tumor stroma, macrophages (iNOS)

**Level of NO production by the tumor:**

eNOS, nNOS: low to moderate

iNOS: moderate to high

(high in an inductive environment)

**Role of NO in tumor biology:**

Genetic make up of the tumor, for example:

wt p53<sup>+</sup>: cytostasis, apoptosis (especially with high NO levels)

p53 null, mutant, inactive: resists NO mediated injury, and uses

NO for tumor progression

**Levels of NO:**

High: cytostasis, apoptosis (in a susceptible genetic make up)

Low to moderate: promotion of tumor progression by increased

angiogenesis, tumor microcirculation and tumor cell invasiveness

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## FIGURE LEGENDS

### Figure 1

Immunostaining patterns for eNOS (A, B, D and E) and iNOS (C) in 12 week old spontaneous (A, B and C) and 3-5 week old transplanted C3L5 (D, E) mammary tumors at the primary (A, C, and D) and metastatic (B, E) sites. Positive immunoreactivity is indicated by brown staining. Blue staining (nuclei, and to a minor extent cytoplasm) is due to counterstaining with hematoxylin.

- A** Spontaneous primary tumor showing eNOS positive as well as eNOS negative tumor cells arranged in pseudoacinar clusters.
- B** Lung metastasis of the tumor A showing strong eNOS positivity in most tumor cells.
- C** Spontaneous primary tumor showing iNOS positive macrophages (<) in the tumor stroma. Tumor cells (\*) were iNOS negative.
- D** Subcutaneous C3L5 primary tumor showing eNOS positivity in nearly every tumor cell.
- E** Strong eNOS positivity is also seen in the majority of C3L5 tumor cells at the site of spontaneous lung metastasis.
- F** A negative control for eNOS staining of the primary C3L5 tumor, in which the eNOS antibody was replaced with an Ig of the same isotype.

### **Figure 2**

Immunofluorescent staining of cultured C3L5 cells for eNOS **(A)** under normal culture conditions, and iNOS **(B)** after 24 h exposure to LPS (10 µg/ml) and IFN-γ (1000 U/ml). Most tumor cells were strongly positive for eNOS **(A)**, and 25-30% of the cells became strongly positive for iNOS **(B)** following induction with LPS and IFN-γ.

### **Figure 3**

Effects of subcutaneous NMMA therapy for 3 days (20 mg/kg/injection, every 8 hr x 10 injections) on days 10-13 after subcutaneous transplantation of  $5 \times 10^5$  C3L5 mammary tumor cells, on mean tumor diameter ( $n = 10-15$ ). There was a significant ( $* p < 0.05$ ) decline in primary tumor size measured on day 14. (*Adapted with kind permission from Orucevic and Lala, Cancer Immunol Immunother, Springer Verlag, 42: 38-46, 1996*)

### **Figure 4**

Effects of subcutaneous NMMA therapy given as two 3 day cycles (days 10-13 and 19-22 after subcutaneous C3L5 tumor transplantation, at the same dose rate as in **Figure 3**), on the number of lung metastatic nodules in mice ( $n = 9$ ) killed on day 22. The therapy resulted in a significant ( $P < 0.05$ ) reduction in spontaneous lung metastasis. (*Adapted with kind permission from Orucevic and Lala, Cancer Immunol Immunother, Springer Verlag, 42: 38-46, 1996*)

### **Figure 5**

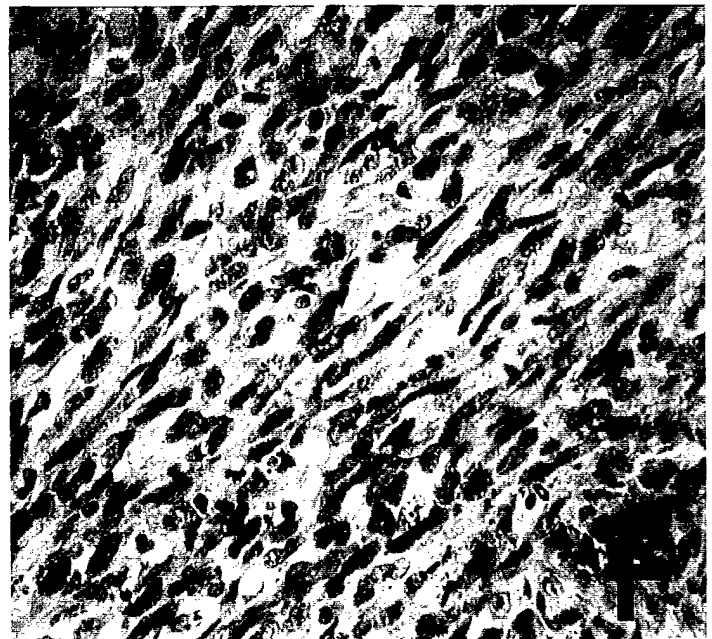
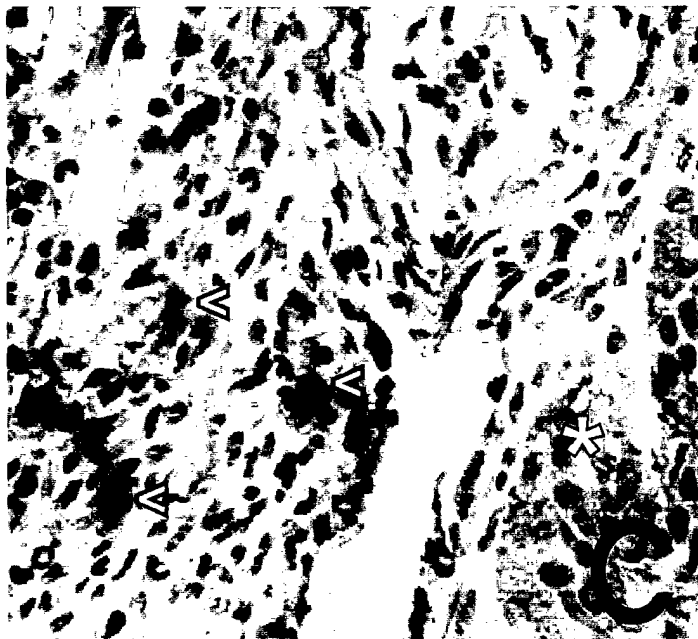
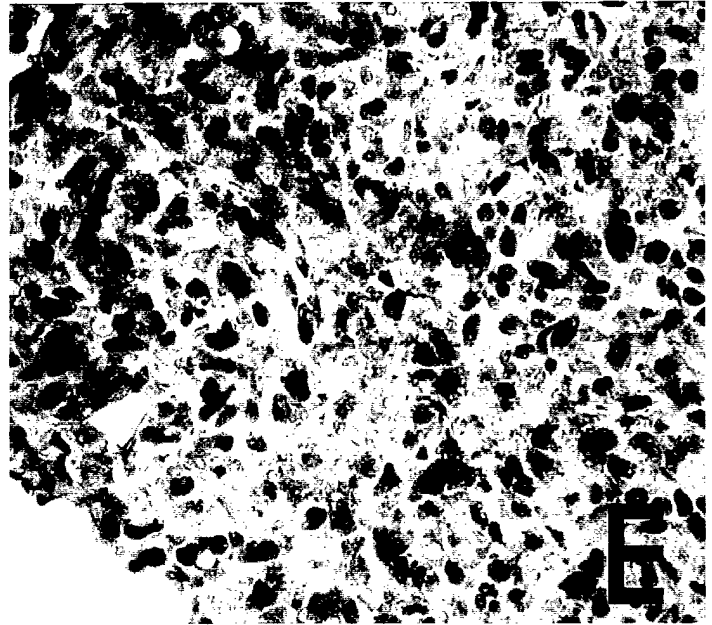
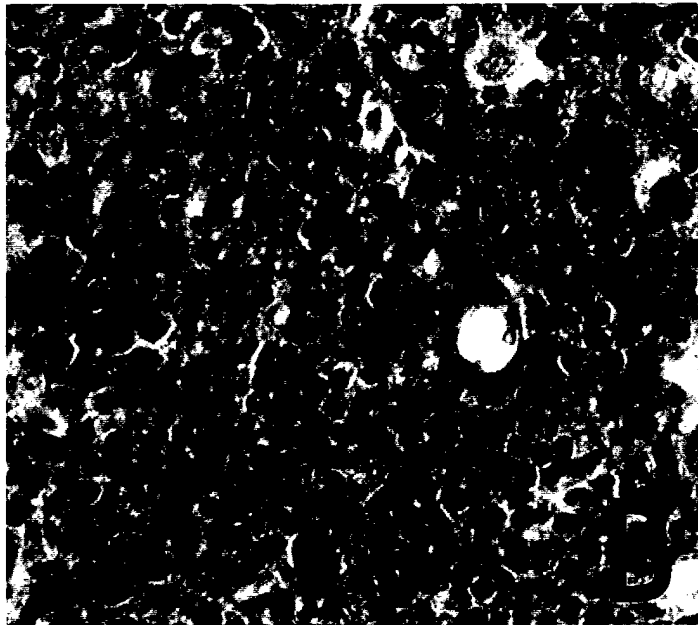
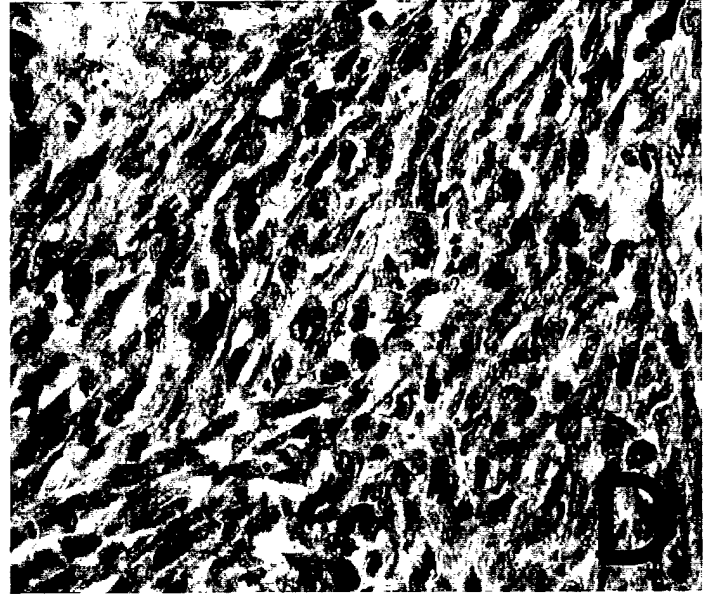
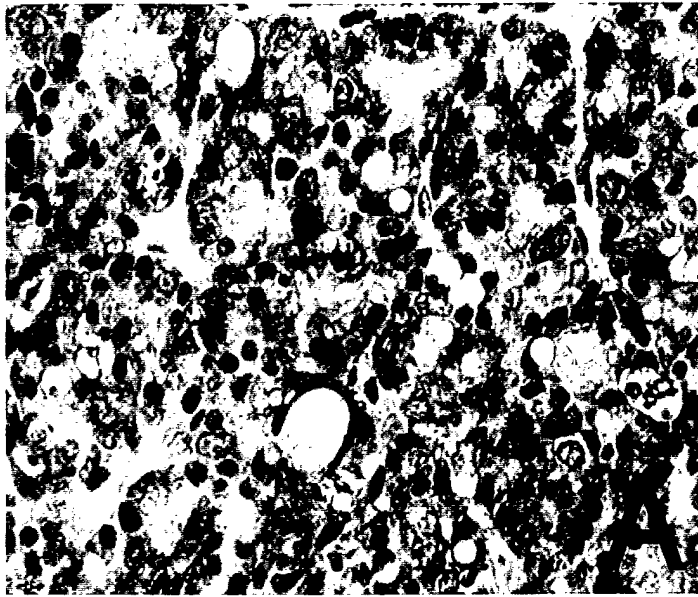
Effects of chronic L-NAME therapy given as two 4 day cycles (days 9-13 and days 19-23) at various concentrations of the drug in the drinking water, on the growth rate of primary tumors following a subcutaneous transplantation of  $2.5 \times 10^5$  C3L5 tumor cells. Animals drank 3-4 ml water/day ( $n = 10-20$ ). The data represent means  $\pm$  SE. There was a dose-dependent decline in tumor growth which was significant at the highest dose (1 mg/ml) throughout the experimental period. (*Adapted with kind permission from Orucevic and Lala, Brit J Cancer 73: 189-196, 1996*)

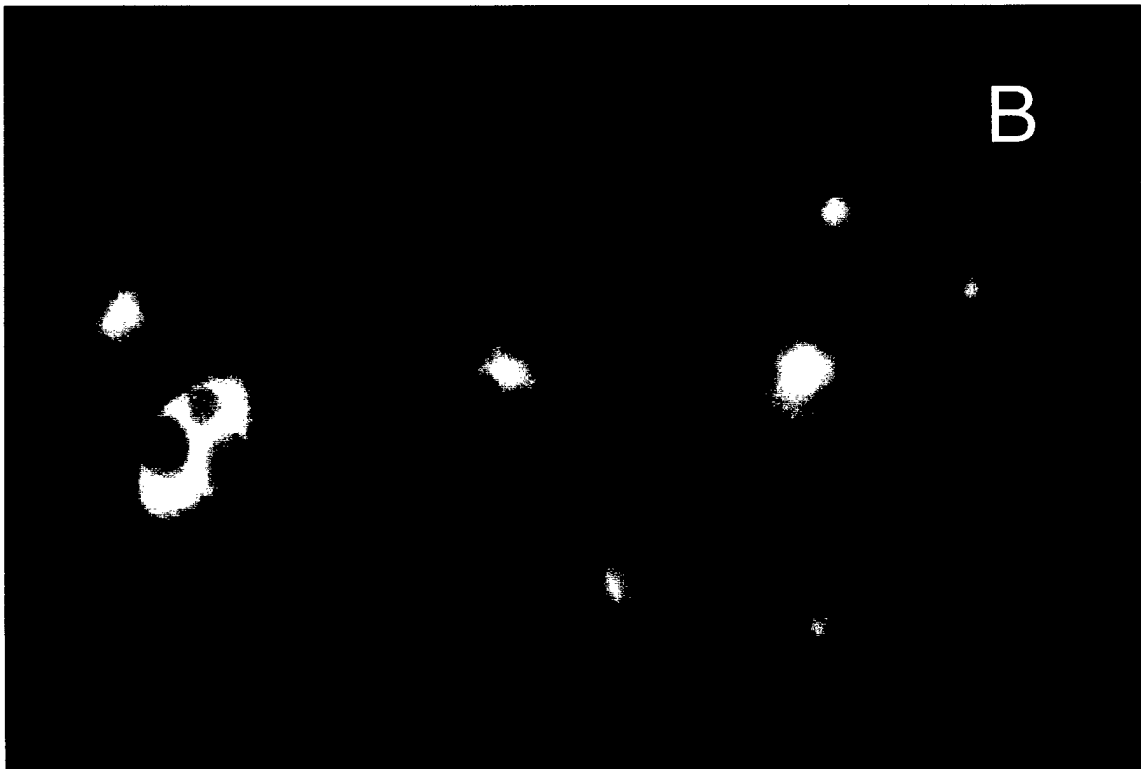
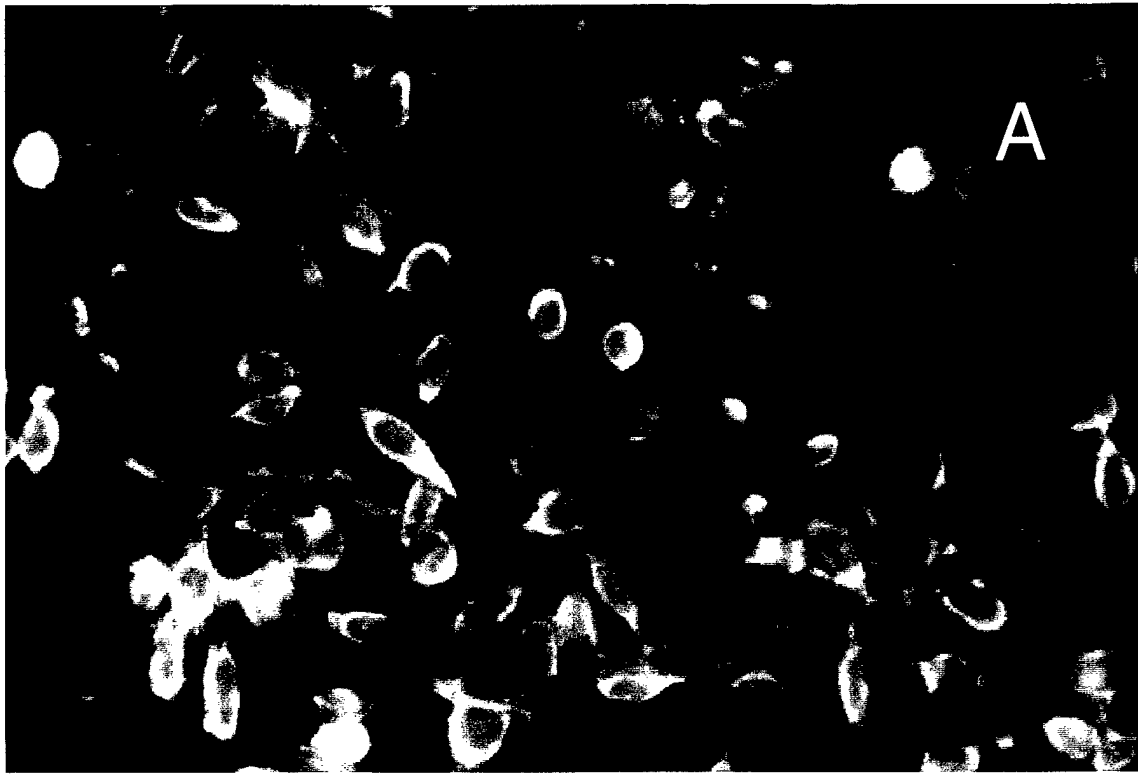
### **Figure 6**

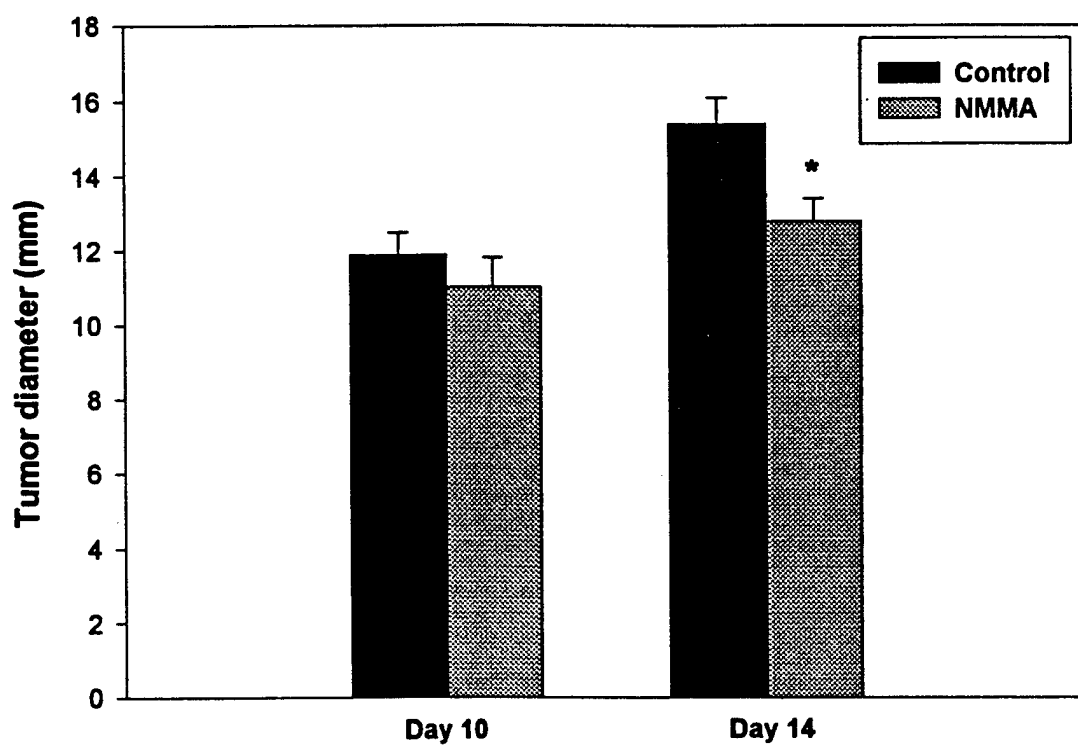
Effects of chronic L-NAME therapy (same dose and schedule as in **Figure 5**) on the number of spontaneous lung metastatic nodules of C3L5 tumor scored on day 23. There was a significant dose dependent reduction in lung metastasis. (*Adapted with kind permission from Orucevic and Lala, Brit J Cancer 73: 189-196, 1996*)

### **Figure 7**

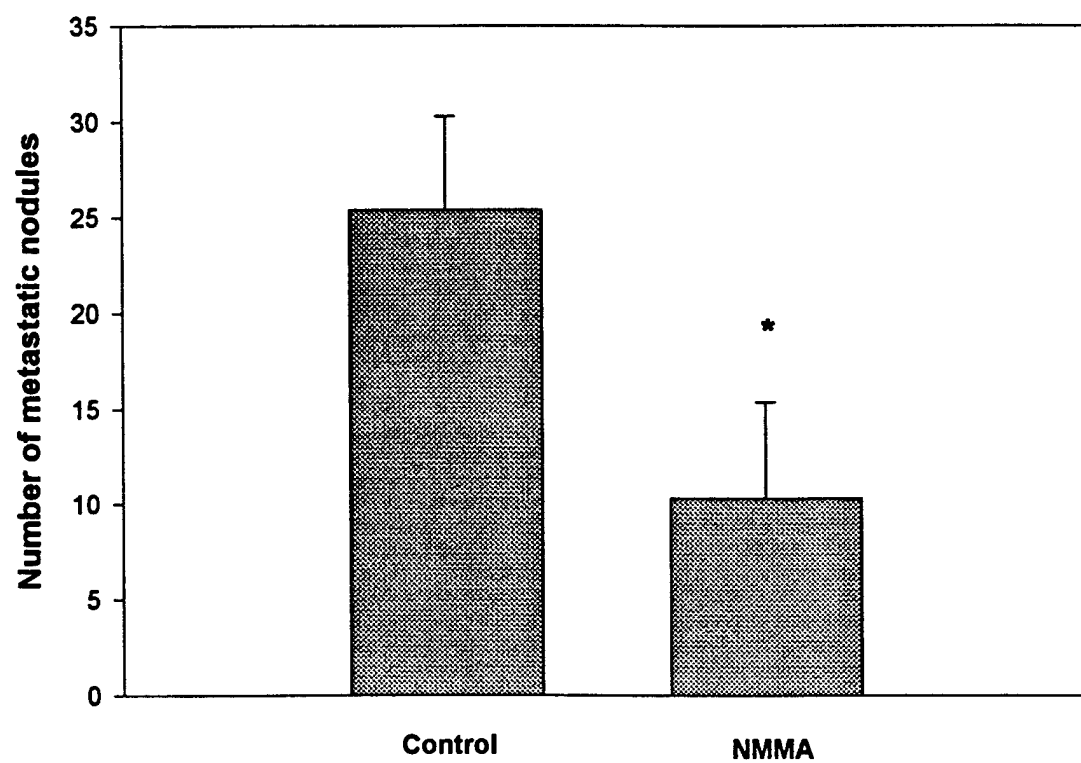
Gross morphology of matrigel implants in (A) matrigel alone implanted in D-NAME treated mice; (B) matrigel inclusive of tumor cells in D-NAME treated mice, and (C) matrigel inclusive of tumor cells in L-NAME treated mice. Note that implant A is mostly avascular, and implant B has grown in size and highly vascular, whereas implant C is smaller in size and less vascular in comparison with B.

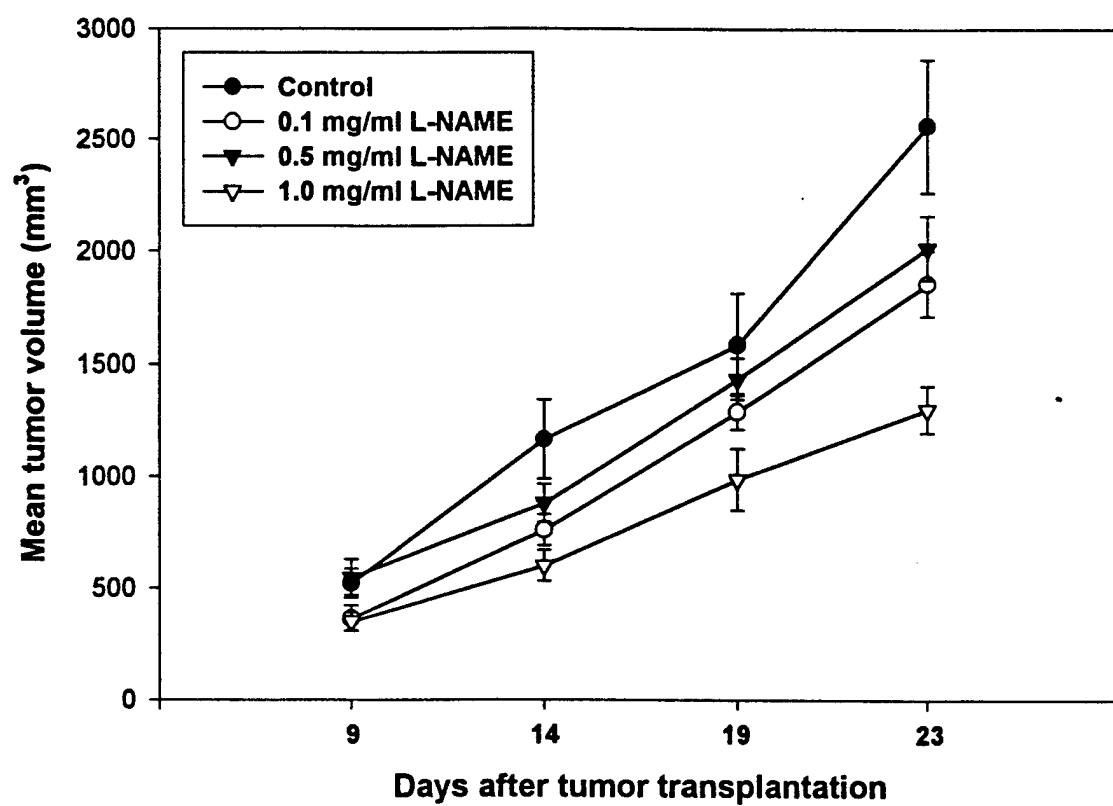


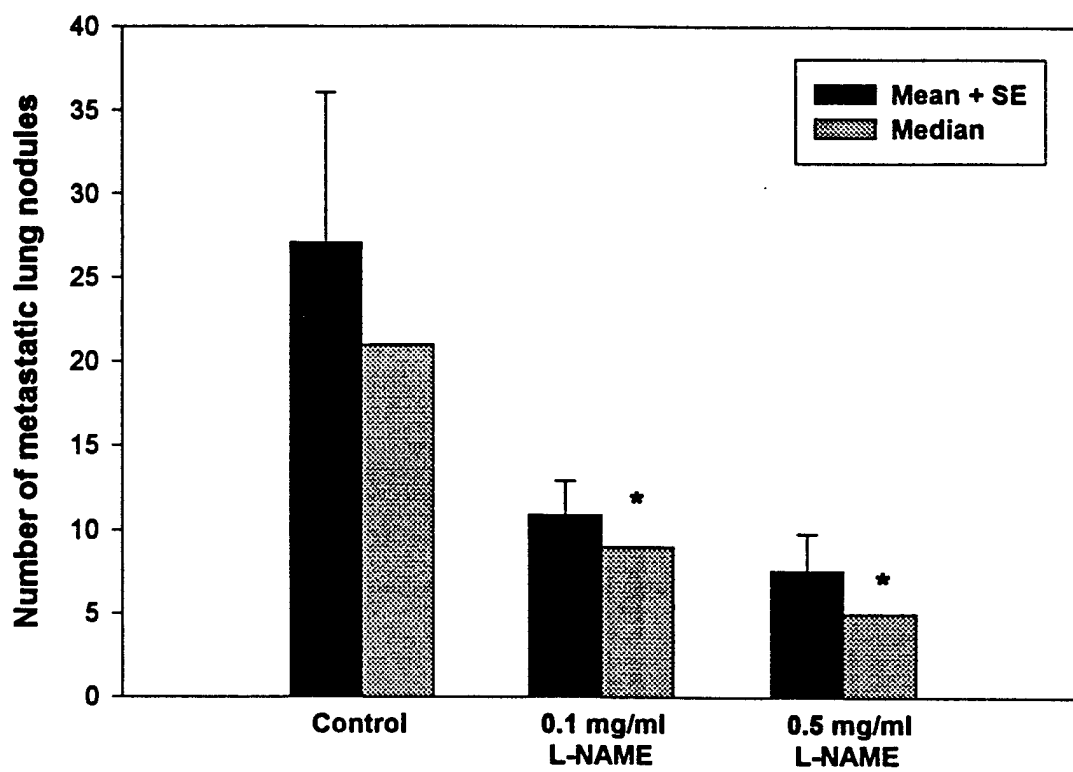


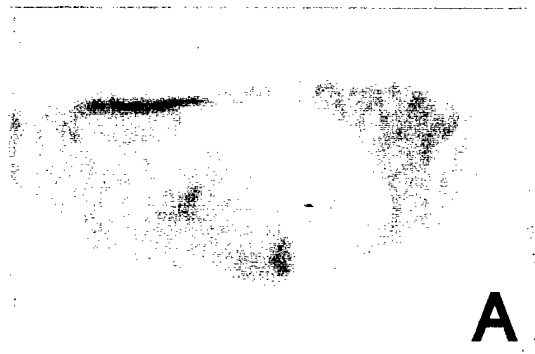








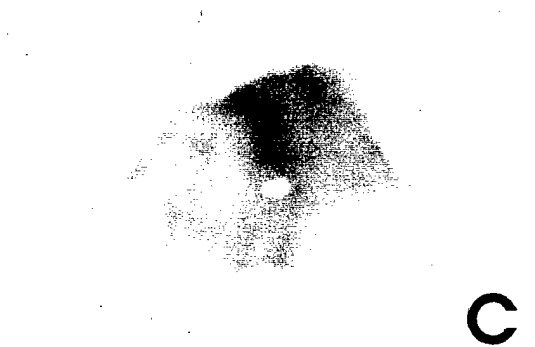




A



B



C

ROLE OF NITRIC OXIDE IN IL-2 THERAPY-INDUCED CAPILLARY LEAK  
SYNDROME

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synthase inhibitors, NMMA, L-NAME, murine mammary adenocarcinoma

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### **Abstract**

Nitric oxide (NO) is a potent short-lived and short range bioactive molecule, which plays a key role in physiological and pathological processes including inflammation and cancer. Detrimental effects of excessive NO production during septic shock have been well recognized. We tested the hypothesis that "capillary leak syndrome" following systemic interleukin-2 (IL-2) therapy resulted from a cascade of events leading to the induction of NO which, directly or indirectly, injured capillaries and caused fluid leakage. Our results provided the first direct evidence that the induction of active NO synthase (NOS) leading to the overproduction of NO is instrumental in IL-2-induced capillary leakage in mice and that successful blocking of this overproduction with chronic oral administration of NOS inhibitors can mitigate this leakage without interfering with the beneficial antitumor effects of IL-2 therapy. NO blocking agents can, in fact, improve IL-2-induced antitumor effector cell activation, as well as tumor regression. In our studies, NO blocking agents alone reduced the growth and metastasis of a murine mammary carcinoma, at least in part, by mitigating the invasion and angiogenesis-stimulating role of tumor-derived NO. Thus, NOS inhibitors may be useful in treating certain tumors and serve as valuable adjuncts to systemic IL-2 based immunotherapy of cancer and infectious diseases.

## I. Introduction

During the last decade, use of systemic interleukin-2 (IL-2) became a major focus of interest in cancer immunotherapy because of IL-2 dependence of all anti-tumor effector cells i.e. T cells [1], natural killer (NK) cells [2] and macrophages [3,4]. The success of high dose IL-2 therapy in metastatic murine cancers [5] soon led to human trials with IL-2 alone or in combination with *ex-vivo* generated lymphokine-activated killer (LAK) cells [6-10], or in combination with chronic indomethacin therapy [11,12] resulting in modest and variable success in renal cell carcinomas and melanomas.

Wide spread clinical use of systemic IL-2 based therapy, has been limited by a major side effect known as "capillary leak syndrome". It is characterized by retention of extravascular fluid, severe hypotension, and multiple organ system dysfunction [13,14], often requiring cessation of IL-2 therapy. This syndrome has been documented in numerous species: humans [13,15,16], mice [17,18], sheep [19-21] and rats [22].

Reported pathophysiological mechanisms underlying this syndrome include damage of endothelial cells by LAK cells [23,24] or NK cells responding to IL-2 [25] or certain IL-2 induced cytokines e.g. interferon (IFN)  $\gamma$  [26] and tumor necrosis factor (TNF)  $\alpha$  [27]. Injury to endothelial cells mediated by these cytokines has been recently linked with nitric oxide (NO) production [28,29], because it was prevented with dexamethasone and inhibitors of NO synthesis. Severe hypotension observed during IL-2 therapy has also been recently attributed to NO production [30,31]. NO is synthesized by many mammalian cells from the amino acid L-arginine, with the help of a family of enzymes called NO synthases (NOS) [32,33]. It is a short lived biological mediator of many

physiological functions. However, sustained overproduction of NO resulting from the induction of the inducible isoform of NOS (iNOS) may have pathological consequences including capillary damage because of cytotoxic action on endothelial cells [28,29]. Vasodilation [34] and systemic hypotension due to NO production can indirectly cause pulmonary hypertension, and the increased pulmonary capillary pressure [13] can lead to fluid leakage in the lungs. Thus, NO may have a major role in the pathogenesis of IL-2 induced capillary leakage. In this paper, we shall briefly review the IL-2 based cancer therapies and possible pathways of IL-2 therapy induced capillary leakage. Since IL-2 therapy induces production of LAK cells [16,35], IFN $\gamma$  [36], TNF $\alpha$  [15,37], and NO [30,31], we shall discuss the independent as well as interdependent roles of these multiple factors. We shall show that NO overproduction occurs at the later part of a cascade responsible for this syndrome and that appropriate administration of NOS inhibitors can not only overcome the syndrome but also improve antitumor effects of IL-2 therapy.

## **II. Systemic IL-2 in tumor immunotherapy**

### **II. A. Biology of IL-2: Tumor therapy with IL-2 as a single agent**

T cell growth factor (later named as IL-2) was initially identified in the supernatant of phytohemagglutinin-stimulated normal human lymphocytes that supported the growth of T cells in culture of normal human bone marrow [38]. IL-2 has since been characterized as a 133 amino acid polypeptide of 15,500 daltons [39, in humans it is encoded by a single gene [40] on chromosome 4. Recombinant IL-2 has been obtained by inserting the IL-2 gene from cultured leukemic cells [41] or from normal peripheral blood lymphocytes [42] in



*Escherichia coli*. This form of recombinant IL-2, although nonglycosylated, has biological activity *in vitro* and *in vivo* identical to that of native IL-2.

The structure of the IL-2 receptor consists of 3 peptide chains ( $\alpha$ ,  $\beta$  and  $\gamma$ ); the genes encoding these chains have been cloned and characterized [43,44]. The  $\alpha$  chain alone provides a receptor of low affinity. Intermediate and high-affinity receptors are produced by  $\beta/\gamma$  heterodimer and  $\alpha/\beta/\gamma$  heterotrimer, respectively, in which the  $\beta$  chain is critical for signal transduction [45]. IL-2 receptor expression has been variably found on the surface of T cells, NK cells [2,46], macrophages [3,4,47], oligodendroglial cells [48], epidermal Langerhans cells [49], B cells [50], and certain tumor cell lines derived from melanomas and squamous cell carcinomas of the head and neck [51].

The tumoricidal potential of all immune effector cells including T cells [1], NK cells [2,52,53], and macrophages [3,4] can be stimulated with IL-2. Lymphocytes cultured in the presence of high dose IL-2 lead to the activation of NK cells and T cells, providing a heterogeneous population of cytotoxic cells with a broad spectrum of antitumor cytotoxicity, known as LAK cells which are capable of killing syngeneic as well as allogenic tumor cells [54]. This knowledge provided the impetus for systemic IL-2 therapy of cancer.

Rosenberg *et al.* [5] were the first to show that systemic administration of IL-2 resulted in regression of pulmonary metastasis in mice by activation of LAK cells *in vivo*. These findings led to the application of IL-2 therapy in human cancers, revealing that highest tumor regression occurred in melanomas and renal cell carcinomas [16,55,56].

## **II. B. Systemic IL-2 in combination with LAK cells in tumor therapy**

Rosenberg's group observed that a combination of systemic IL-2 therapy with infusion of LAK cells generated *in vitro* had significantly higher antitumor activity in mice than IL-2 therapy alone [57,58]. Intravenous IL-2 therapy in combination with LAK cells was then applied to treat human patients with solid tumors. Autologous lymphocytes were obtained from cancer patients by repeated leukaphereses, cultured in the presence of IL-2 to generate LAK cells, and reinfused into the patients together with IL-2 [16]. This treatment resulted in the regression of tumors in some patients for whom no other effective therapy was available [35,59]. However, it was soon apparent that the therapeutic benefit derived from this combination therapy was not greater than that from IL-2 therapy alone [7].

## **II. C. IL-2 in combination with tumor infiltrating lymphocytes in tumor therapy**

Lymphocyte-trafficking studies with radiolabeled LAK cells generated from blood or splenic lymphocytes showed that LAK cells did not localize at tumor metastatic sites but were trapped in the lungs and later in the liver. However, lymphocytes retrieved from the tumor and expanded with IL-2 showed some selectivity for migration to the tumor metastatic site after infusion *in vivo* [60]. These "tumor infiltrating lymphocytes" (TIL) were expanded *in vitro* [61] for adoptive transfer. When TILs were infused along with IL-2 in patients with melanoma or renal cell carcinoma, responses were higher relative to IL-2 treatment alone or IL-2 combined with LAK cells [61-63].

## **II. D. IL-2 in combination with chronic indomethacin therapy in tumor treatment**

Lala et al. [64] observed that natural killer cells were progressively inactivated in the tumor-bearing host with increasing tumor burden. This inactivation was caused by a high level of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) produced by host macrophages [65] as well as certain tumor cells [66]. PGE<sub>2</sub> has been shown to suppress lymphocyte proliferation [67] and activation of all antitumor killer cell lineages [4]. These effects, at least in part, explained the promotion of metastatic ability of tumors by PGE<sub>2</sub> [68]. The PGE<sub>2</sub>-mediated inactivation of effector cells was attributed to inhibition of IL-2 production [69] and a down regulation of IL-2 receptors on the surface of all killer cell lineages [70].

Based on these findings, Lala's group started an immunotherapy protocol combining systemic IL-2 with chronic oral administration of indomethacin [71], a drug that inhibits prostaglandin production [67]. Chronic indomethacin therapy had antitumor and antimetastatic effects [72,73], and substantially restored natural killer cell function [73] in murine tumor models. However, this therapy alone was unable to eradicate advanced metastases [71], possibly because of inadequate IL-2 production *in vivo*. Chronic indomethacin therapy (given in the drinking water) when combined with systemic injections of IL-2 resulted in permanent regression of B16F10 melanoma metastases in the lungs of a large proportion of animals [74]. Reactivation of AGM-1+ and Thy-1+/- killer lymphocytes *in situ* accounted for the therapeutic benefit, since depletion of these cells *in vivo* abrogated the therapeutic effects. Similar eradication of metastases was also achieved in C3-L5 mammary adenocarcinomas [75] and human melanomas grown in nude mice [76]. This combination therapy was then applied in a phase 2 human trial of advanced melanoma and renal cell carcinoma patients resulting in good objective responses [11,12], comparable

with the higher ranges in the success rates reported in the other IL-2 based therapy trials [77]. Interestingly, the toxicity was manageable in a general oncology ward without the need for vasopressor agents often used in other IL-2 trials [77], and some melanoma patients responded to indomethacin therapy alone [78].

### **III. Capillary leak syndrome due to systemic IL-2 therapy**

Initially, it was believed that the efficacy of IL-2 in the therapy of cancer improved as a function of the IL-2 dose administered [56,59]. Although true for animal models, this association was very weak in a controlled study in renal cell carcinoma patients, receiving high or low-dose of intravenous IL-2 [14], and undetectable in a study in renal cell carcinoma patients using indomethacin in combination with IL-2 [11,12,79]. However, dose-related toxicity was observed in most trials and still remains a major obstacle to systemic IL-2 - based therapy. Capillary leak syndrome is the most serious side effect of moderate to high doses of IL-2 observed in many species [13,15,18,20,21,22]. There is an increase in microvascular permeability causing marked accumulation of extravascular fluid in all organ systems and hypotension, often requiring treatment with intravenous fluids and vasopressor agents [81]. Retention of extravascular fluid results in rapid weight gain of up to 20%, manifested by peripheral edema, pleural effusion and ascites [13,14]. Occasionally, life threatening pulmonary edema, respiratory or cardiac failure, and neurological abnormalities resulting in coma (due to edema of the brain) may develop during IL-2 therapy, requiring cessation of the therapy [13,14]. Interestingly, symptoms of capillary leakage begin to reverse within 24 h of cessation of IL-2 therapy and

usually completely disappear within a few days [14]. Capillary leak syndrome has been observed with IL-2 therapy alone and IL-2 therapy in combination with LAK cells or TIL. A less severe form of the syndrome has also been noted with IL-2 therapy in combination with indomethacin therapy.

Several studies have combined IL-2 with other agents to ameliorate the capillary leakage. However, the added drugs also blocked or reduced the beneficial antitumor effects of IL-2. Corticosteroids [17], which suppress inflammatory responses and induction of NO [82], and asialo-GM-1 antibody, which depletes LAK cells [18], both fall in this category. Puri *et al.* [83] reported that IL-1 $\alpha$  reduced IL-2-induced capillary leakage but did not improve animal survival. Welbourn *et al.* [84] reported that certain cyclopeptides (e.g. antamanide and phalloidin), reduced IL-2-induced edema in the rat, presumably by causing cytoskeletal changes in neutrophils with consequent suppression of endothelial injury by thromboxane B<sub>2</sub>. Influence of these agents on the antitumor effect of IL-2 remains unknown. Further studies were therefore required to identify substances that can ameliorate capillary leakage without compromising the anti-tumor effects of IL-2.

Based on the observations that systemic IL-2 therapy in combination with chronic indomethacin therapy in advanced melanoma and renal cell carcinoma patients [11,12] was associated with less severe IL-2 toxicity than reported in the case of other IL-2 trials, we tested in a mouse model whether PGE<sub>2</sub> played any role in the IL-2-induced capillary leakage [85,86]. Our results revealed that addition of chronic indomethacin treatment markedly improved the antitumor effects of IL-2 therapy, but was unable to ameliorate the IL-2-therapy - induced capillary leakage.

### **III. A. Possible mechanisms of IL-2 induced capillary leak syndrome**

At least one or both of two conditions must be satisfied to cause capillary leakage: capillary endothelium must be damaged or the capillary pressure increased. Five mechanisms have been proposed by which IL-2 therapy can induce capillary leakage.

- (i). IL-2 induces LAK cells to adhere to and later damage endothelial cells.
- (ii). IL-2 induces NK cells to adhere to and damage endothelial cells.
- (iii). Endothelial cells are damaged by  $\text{TNF}\alpha$  produced by IL-2-activated leukocytes.
- (iv). Changes in endothelial cell architecture are caused by  $\text{IFN}\gamma$  produced by IL-2-activated leukocytes.
- (v). IL-2 directly or indirectly induces NO production which is toxic for endothelial cells. In addition, NO, because of its vasodilatory role, leads to systemic hypotension which indirectly causes pulmonary hypertension resulting in an increase in pulmonary capillary pressure and thus pulmonary edema.

#### **III. A. 1. LAK cells and capillary leak syndrome**

LAK cells have been shown to adhere to endothelial cells and cause their lysis *in vitro* [23,24,87]. Kotasek *et al.* [23] proposed that the dense granules secreted by LAK cells, which contain serine esterase I (an enzyme with high proteolytic and cytolytic activity), caused breaches in the endothelial cell membranes. Observations on cultured endothelial cells led Savion *et al.* [88] to propose that LAK cells migrated through and ruptured endothelial cell tight junctions. Once they reached the basement membrane and the subendothelial

matrix, LAK cells would degrade the matrix by producing matrix-degrading enzymes. These events, with or without endothelial cell lysis, would result in capillary leakage. The hypothesis of LAK cell mediated capillary injury is substantiated by the findings that LAK cell depletion *in vivo* by treatment with asialo-GM-1 antibody in mice ameliorated IL-2 therapy induced capillary leakage [18]. However, this treatment also abrogated antitumor effects of IL-2.

### **III. A. 2. NK cells and capillary leak syndrome**

Aronson *et al.* [25] showed that IL-2 can induce NK cells to adhere to human endothelial cells in culture. These authors implied that vascular leakage induced by IL-2 resulted from NK cell mediated endothelial cell injury. However, there has been no direct evidence of NK cells causing endothelial cell damage *in vivo*.

### **III. A. 3. TNF $\alpha$ and capillary leak syndrome**

IL-2 therapy activates leukocytes (monocyte-macrophage in particular) to produce TNF $\alpha$  [15,37]. Several authors have reported controversial findings about the ability of TNF $\alpha$  to damage endothelial cells. Collins *et al.* [89] reported that TNF $\alpha$  activated human endothelial cells to express class 1 HLA antigen, suggesting that TNF $\alpha$  made them prone to cytolytic T lymphocyte mediated injury. Kahaleh *et al.* [27] showed that TNF $\alpha$  inhibited endothelial cell growth in culture, and at high concentrations, induced endothelial cell lysis.

In 1990, Doukas and Pober [90] reported that TNF $\alpha$  led to endothelial cell "activation", which was enhanced further by IFN $\gamma$ . "Activation" was indicated by appearance of new morphologic, antigenic and functional characteristics of

endothelial cells. Increases in specific endothelial cell surface molecules like ELAM-1 (endothelial leukocyte adhesion molecule 1) or ICAM (intercellular cell adhesion molecule) were observed by these authors after stimulation by  $\text{TNF}\alpha$  and  $\text{IFN}\gamma$ . IL-6 production in response to  $\text{TNF}\alpha$  was observed by Leewenberg *et al.* [91]. Endothelial cell activation and increased adhesiveness for leukocytes were implied to play a role in increased capillary permeability.

In contrast, Mier *et al.*, [92] reported that  $\text{TNF}\alpha$  and  $\text{IFN}\gamma$  activated endothelial cells and increased the binding of CD16+ lymphocytes to endothelial cells in culture, but that the lymphocyte binding was not responsible for increased capillary permeability. In fact, these authors reported that  $\text{TNF}\alpha$  and  $\text{IFN}\gamma$  protected endothelial cells from LAK cell-mediated injury.

#### **III. A. 4. Changes of endothelial architecture induced by $\text{IFN}\gamma$**

$\text{IFN}\gamma$  appears in the blood of cancer patients within 6 hours after administration of IL-2 [15]. Cytotoxic activity of  $\text{IFN}\gamma$  is well known, but its possible role in IL-2-induced capillary leak syndrome remains obscure. Montesano *et al.* [26] showed that certain lymphokines could alter human endothelial cell architecture *in vitro*. IL-2 had no effect, and  $\text{IFN}\gamma$  had only a marginal effect. Combination of IL-1 and  $\text{IFN}\gamma$  completely changed the appearance of endothelial cells. They became elongated with many "dendrite like" processes, and there were changes in cytoskeletal structure.

A causal relationship between changes in endothelial cell morphology induced by these lymphokines *in vitro* and in the capillary leak syndrome *in vivo* remains to be established. In fact, Puri *et al.* [83] reported that administration of recombinant IL-1 *in vivo* reduced IL-2 induced vascular leakage in the lungs of



mice. These authors could not explain the failure of IL-1 to increase survival of mice treated with IL-2 or with IL-2 and IFN $\gamma$ .

### **III. A. 5. NO and capillary leak syndrome**

Based on the findings that NO can be produced by activated macrophages after treatment with endotoxin, IFN $\gamma$  or certain other cytokines, [93,94], Kilbourn and Belloni [95] investigated the effects of IFN $\gamma$ , TNF $\alpha$ , IL-1, IL-2 and endotoxin on the production of NO by endothelial cells. They showed that culture of murine brain endothelial cells produced NO in response to various combinations of cytokines. They speculated that endothelium-derived NO played a role in the development of hypotension in patients treated with IL-2 or TNF $\alpha$ . In support of this hypothesis, Kilbourn *et al* showed that therapy with NG-Methyl-L-Arginine (NMMA, an inhibitor of NO synthesis) protected dogs against hypotension induced by TNF $\alpha$  and endotoxin [96,97], as well as IL-2 [80].

Increased levels of the final metabolites of NO (nitrates and nitrites) [82,98] have been reported in human cancer patients receiving IL-2 therapy [30,31,99]. NO induction may be an indirect result of IL-2 therapy due to an induction of IFN $\gamma$  and TNF $\alpha$  [15,37]. Endothelial injury mediated by both of the cytokines has been linked with NO production [28,29]. NO can contribute to capillary leakage by direct or indirect mechanisms. First, NO has been shown to mediate cytotoxicity in endothelial cells [28,29] and thus cause a loss of integrity of the capillary lining. Second, high NO levels can indirectly enhance the capillary leakage in the lungs. It causes systemic hypotension [100] which in turn can indirectly cause pulmonary hypertension and thus increased pulmonary capillary pressure leading to further fluid leakage in the lungs.

#### **IV. Capillary leak syndrome results from numerous simultaneous or sequential events induced by IL-2 therapy: a hypothesis**

In view of the literature reviewed earlier, it is reasonable to suggest that an increase in capillary permeability is caused by multiple factors initiated by high doses of IL-2. These factors operate by causing damage to capillary endothelial cells and/or by increasing capillary pressure. Capillary leak syndrome may result from numerous simultaneous or sequential events: 1) IL-2 induces LAK cell activation *in vivo* and promotes their adhesion to and subsequent cytotoxicity to endothelial cells; 2) IL-2 induces high levels of IFN $\gamma$  which can change the cytoarchitecture of endothelial cells, making the endothelial lining more prone to leakage; 3) high levels of TNF $\alpha$  produced by IL-2-activated leukocytes induces endothelial cell activation and adhesiveness for leukocytes and may play a role in increased capillary permeability; 4) NO induction at high levels remains at the end of the cascade of events induced by IL-2 therapy (e.g. production of TNF $\alpha$  and IFN $\gamma$ ) and plays a major role in capillary leakage both directly and indirectly, as discussed above.

#### **V. Evidence for the central role of NO in the development of IL-2 therapy-induced capillary leakage in mice and its mitigation with NOS inhibitors**

We conducted a series of studies to examine the role of NO in the pathogenesis of capillary leakage resulting from systemic IL-2 therapy in healthy and mammary adenocarcinoma-bearing C3H/HeJ mice. We measured IL-2

therapy-induced capillary leakage (pleural effusion, pulmonary edema and water retention in the spleen and the kidneys), NO production *in vivo* and the influence of treatment with NOS inhibitors (NMMA and N<sup>G</sup>-Nitro L-Arginine methyl ester - L-NAME) on these parameters. Influence of these two inhibitors on IL-2 therapy-induced regression of the primary tumors and their lung metastases was also examined. In addition, the effects of these NOS inhibitors alone on mammary tumor growth and metastases were evaluated. Since L-NAME potentiated tumor-reductive effects of IL-2 therapy simultaneously with a reduction of IL-2-induced NO production *in vivo*, further experiments were designed to test whether L-NAME had a potentiating effect on IL-2-induced activation of antitumor effector cells *in vivo* and *in vitro*. This was tested by measuring antitumor cytotoxicity of splenocytes of healthy or tumor-bearing mice subjected to IL-2 ± L-NAME treatment *in vivo* and *in vitro*.

We initially tested whether treatment with NMMA can ameliorate IL-2 therapy-induced capillary leak syndrome in healthy or tumor-bearing mice without compromising the antitumor effects of IL-2 therapy [101]. We found that intraperitoneal IL-2 therapy caused substantial capillary leakage, both in healthy and tumor-bearing mice, as well as a substantial rise in NO production *in vivo* (measured in the serum and pleural effusion) in an IL-2 dose-dependent manner. Subcutaneously administered NMMA, when combined with IL-2 therapy, failed to ameliorate IL-2-induced capillary leakage in both groups of mice, and was also inadequate in significantly reducing IL-2 induced rise in NO production *in vivo*. It did not compromise anti-tumor effects of IL-2. In mammary adenocarcinoma bearing mice, subcutaneous NMMA therapy alone reduced

tumor growth, spontaneous pulmonary metastasis and tumor-induced pulmonary edema.

This prompted us to test the effects of continuous oral administration of NMMA in healthy mice subjected to IL-2 therapy. A substantial drop in NO production and capillary leakage was noted in these mice [101]. Since NO-blocking agents protected against IL-2-induced hypotension [80,100], it was reasonable to expect that NMMA should also prevent IL-2 induced fluid leakage. It was evident that NMMA fulfilled this expectation only when given orally but not subcutaneously. This may be because the continuous oral administration of the drug was effective in blocking the rise in serum NO levels induced by IL-2 therapy, whereas the subcutaneous administration, in spite of repeated delivery, was inadequate in fully blocking NO production [101]. We suggested that the route of administration, as well as scheduling, were important determinants of therapeutic efficacy of NO inhibitors in the mitigation of IL-2 induced capillary leakage. This contention was supported by our findings that another NOS inhibitor, L-NAME also succeeded in mitigating IL-2 induced capillary leakage in healthy mice when given orally, but the benefits were only partial when given subcutaneously [102]. Similarly, attenuation of IL-2-associated capillary leakage was observed in another murine model by Samlowski *et al.* [103] when an NO inhibitor was given continuously in an osmotic minipump, whereas no effect on the IL-2-induced capillary leakage was noted by Leder *et al.* [104] when the NO inhibitor was given subcutaneously.

We did not test oral NMMA therapy in tumor-bearing mice, since L-NAME, another potent and less expensive NO inhibitor was soon available. Thus, we first tested whether L-NAME given chronically in the drinking water was

effective in preventing capillary leakage induced by IL-2 therapy in healthy mice. We found that L-NAME was effective in preventing capillary leakage (pleural effusion - Figure 1, pulmonary edema - Figure 2 and water content of the spleen - not shown) induced by IL-2 therapy in healthy mice, and reduced IL-2-induced mortality when the IL-2 dose was not very high [102]. NO production appeared to be a strong determinant of the severity of this syndrome, because L-NAME treatment had a parallel effect in ameliorating the IL-2-induced capillary leakage and rise in NO production (Figure 3). A subsequent study [105] was designed in healthy C3H/HeJ mice to (a) identify the tissue source of NOS activity and NOS protein induced by IL-2 therapy; (b) identify the histological nature of structural damage to the lungs during IL-2 therapy-induced pulmonary edema and (c) test whether the addition of L-NAME therapy reduced the increase in NOS activity and IL-2-induced structural damage to the lungs. Morphological studies revealed that IL-2 therapy led to the induction of iNOS protein in numerous tissues, including the vascular endothelium, muscles of the anterior thoracic wall and splenic macrophages [105]. Biochemical studies revealed a positive association of high NOS activity in the lungs and the anterior thoracic wall with the presence of pulmonary edema, pleural effusion and structural damage to the lungs and its capillaries in IL-2 treated mice. Addition of L-NAME completely abolished the NOS activity, but not necessarily iNOS expression. It also reduced IL-2-induced pulmonary edema and pleural effusion, and significantly restored structural integrity of the lungs identified by light and electron microscopy (Figure 4) [105]. Thus, high tissue activity of IL-2-induced iNOS enzyme played a crucial role in the pathogenesis of IL-2 induced capillary leak syndrome.

Next, we tested whether L-NAME can prevent IL-2-induced capillary leakage in mammary adenocarcinoma bearing mice without compromising the therapeutic benefit of IL-2 [106]. In tumor-bearing mice, oral L-NAME therapy alone produced significant anti-tumor and anti-metastatic effects, similar to the effect noted earlier with NMMA therapy. L-NAME in combination with IL-2 therapy succeeded in ameliorating IL-2-induced as well as tumor-induced capillary leakage in tumor-bearing mice (Figure 5), and potentiated the tumor-reductive function of IL-2 [106]. Therefore, we tested whether a potentiation of IL-2-induced tumor regression by L-NAME therapy can be explained by a potentiation of LAK cell activation [107]. We found that L-NAME treatment *in vivo* as well as *in vitro* markedly stimulated IL-2 induced generation of antitumor cytotoxicity of splenocytes of healthy (Figure 6) as well as mammary adenocarcinoma-bearing mice (not shown), concomitant with a drop in IL-2-induced NO production *in vivo* and *in vitro*. These results revealed that the IL-2-induced increase in NO production had a compromising effect on optimal LAK cell activation, which can be overcome by NO inhibition with L-NAME therapy [107].

The above results provided the first direct evidence that NO is instrumental in IL-2-induced capillary leakage and that an NO blocking agent such as L-NAME can mitigate this leakage without interfering with the beneficial anti-tumor effects of IL-2 therapy. We also found that NO blocking agents alone can reduce tumor growth and spontaneous metastasis in this mammary tumor model in which tumor cells express eNOS. When combined with IL-2, NOS inhibitors improved IL-2 induced antitumor cytotoxicity, as well as tumor regression. Thus, NO blocking agents may be useful in treating tumors

producing NO and serve as valuable adjuncts to IL-2 based therapies of cancer and infectious diseases.

Toxic side effects of systemic high dose IL-2 therapy, including capillary leakage, have recently forced investigators to seek alternate forms of IL-2 delivery, including gene therapy. These efforts have so far been less than promising in limited human trials. We propose that more research should be invested into combination therapies for achieving the dual benefit of amelioration of IL-2 toxicity and augmentation of the antitumor efficacy of systemic IL-2 therapy. A recent report [108] indicates that induction of oxygen-free-radicals may represent an additional arm of endothelial injury caused by IL-2 therapy, since treatment with dimethylthiourea (a scavenger of oxygen-free-radicals) attenuated IL-2 therapy- induced capillary leakage. We suggest that formation of peroxynitrite, a potent endotheliotoxic molecule, due to combination of NO with superoxide may be the strongest mediator of IL-2 induced capillary damage. It remains to be seen whether combination therapies designed to block both NO and superoxides can provide better means of controlling IL-2 toxicity and improving antitumor effects of IL-2.

#### **Key unanswered questions**

1. Can more specific iNOS inhibitors such as L-N<sup>6</sup>-(1-Iminoethyl)-lysine hydrochloride (NIL) [109] or 1400W [110] given continuously in an osmotic minipump provide better protection from IL-2 induced mortality in mice than L-NAME?

2. How does IL-2 therapy-induced NO mediate the damage to endothelial cells: by direct NO-mediated apoptosis, or endothelial injury caused by an over production of peroxynitrite, or both?
3. Will the combination of an iNOS inhibitor and an oxygen - free - radical - scavenger (such as dimethylthiourea) prove to be superior to either of these agents alone in preventing IL-2 induced capillary leakage and mortality ?

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### Figure legend

#### **Figure 1.** Pleural effusion after IL-2 and L-NAME therapy in healthy mice.

Data represent mean  $\pm$  SE (n=5). \* indicates significant difference from IL-2 treatment ( $p<0.05$ ).

L-NAME (0.01, 0.1 or 1 mg/ml of drinking water) significantly ( $p<0.05$ ) reduced IL-2 (35,000U/inj i.p., every 8 h , 10 inj. total) induced pleural effusion. Significant reduction ( $p<0.05$ ) of pleural effusion induced by lower IL-2 dose (15,000 U/inj or 7,500 U/inj) was noticed only with high L-NAME dose (1 mg/ml of drinking water). Neither control (untreated) nor L-NAME alone treated mice showed any pleural effusion (data not shown). *Reproduced with kind permission from Orucevic and Lala, J Immunother., 18: 210-220, 1996 Lipincott Raven Publishers.*

**Figure 2.** Water content of the lungs after IL-2 and L-NAME therapy in healthy mice. Data represent mean  $\pm$  SE (n=5). \* indicates significant difference from control ( $p<0.05$ ). \*\* indicates significant difference from IL-2 treatment ( $p<0.05$ ).

IL-2 (35,000 U/inj or 15,000 U/inj i.p., every 8h, 10 inj total) induced pulmonary edema was significantly ( $p<0.05$ ) reduced with addition of L-NAME (0.1 mg/ml or more) in a dose dependent manner, being abolished at a dose of 1 mg/ml. Low dose of IL-2 (7,500 U/inj) also induced pulmonary edema, but addition of L-NAME did not have any significant effect. *Reproduced with kind permission from Orucevic and Lala J Immunother., 18: 210-220, 1996 Lipincott Raven Publishers.*

**Figure 3.** Nitrite + nitrate levels in the pleural effusion after IL-2 and L-NAME therapy in healthy mice ( $10^{-6} \times M = \mu M$ ). Data represent mean  $\pm$  SE (n=3-5, each done in duplicate). \* indicates significant difference from IL-2 treatment ( $p < 0.05$ ). IL-2 (15,000 U/inj or 35,000 U/inj, i.p., every 8 h, 10 inj. total) induced dose dependent increases in nitrite + nitrate levels in the pleural effusion were significantly ( $p < 0.05$ ) reduced with addition of L-NAME (0.1 mg/ml or more). L-NAME did not have any effects on the nitrite + nitrate levels induced by low IL-2 dose (7,500 U/inj). *Reproduced with kind permission from Orucevic and Lala J Immunother., 18: 210-220, 1996 Lipincott Raven Publishers.*

**Figure 4.** Ultrastructure of the lungs of mice given IL-2 or IL-2 + L-NAME therapy. a = control; b = IL-2; c = IL-2 + L-NAME; magnification  $\times 20,000$ . Therapies were given in the following manner: IL-2 was given in a dose of 15,000 U/inj, i.p., every 8h, 10 injections total; L-NAME was given in drinking water starting 1 d before IL-2 therapy.

Basement membrane is thick (  $\leftarrow$  ) and discontinuous in IL-2 treated mice. Endothelial as well as pneumocyte type I are severely damaged. There is also swelling of endothelial cells as well as pneumocyte type I. > indicates an area of blood-air barrier showing such damage. Addition of L-NAME therapy restored the ultrastructural integrity of alveoli and endothelium. Basement membrane is continuous and thin at the thin part of the capillary ( \* ) in IL-2 + L-NAME treated animals. Endothelial cells, although in some cases remain swollen, are never detached from their basement membrane in these mice. *Reproduced with kind permission from Orucevic et al., Lab. Investigation, 76 (1): 53-65 , 1997, The United States and Canadian Academy of Pathology Inc.*

**Figure 5.** Water content of the lungs and spleen after IL-2 and L-NAME therapy in tumor-bearing mice. (left: lungs, right: spleen). Data represent mean  $\pm$  SE (n = 10). Therapies were given in the following manner: IL-2 was given i.p. in a dose of 15,000 U/inj, every 8h, 10 injections total, started 10 d after sc. inj of 250,000 C3-L5 mammary adenocarcinoma cells; L-NAME was given in drinking water starting on d 9 after tumor inoculation. \* Addition of L-NAME significantly ( $p < 0.05$ ) reduced IL-2 induced pulmonary edema after the first round of therapy.

\* Addition of L-NAME significantly ( $p < 0.001$ ) decreased IL-2 induced water retention in the spleen in a dose dependent manner after the first round of therapy. *Reproduced with kind permission from Orucevic and Lala, Br. J. Cancer, 73: 189-196, 1996, Stockton Press, Hampshire, UK.*

**Figure 6.** *In vivo* killer cell generation in healthy mice after IL-2  $\pm$  L-NAME therapy. Data represent mean  $\pm$  SE (every effector : target ratio done in triplicate). \* IL-2 therapy significantly ( $p < 0.05$ ) improved splenocyte cytotoxicity (all three effector : target ratios combined) against NK sensitive -YAC-1 target and NK resistant - C3-L5 target. \*\* Addition of L-NAME therapy significantly ( $p < 0.05$ ) enhanced IL-2 induced splenocyte cytotoxicity (all three effector : target ratios combined) against NK sensitive and NK resistant targets. *Reproduced with kind permission from Orucevic and Lala, Cellular Immunol., 169: 125-132, 1996, Academic Press, New York, USA.*

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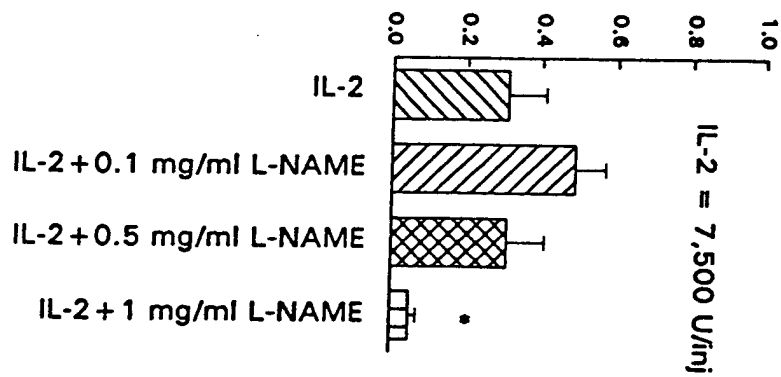
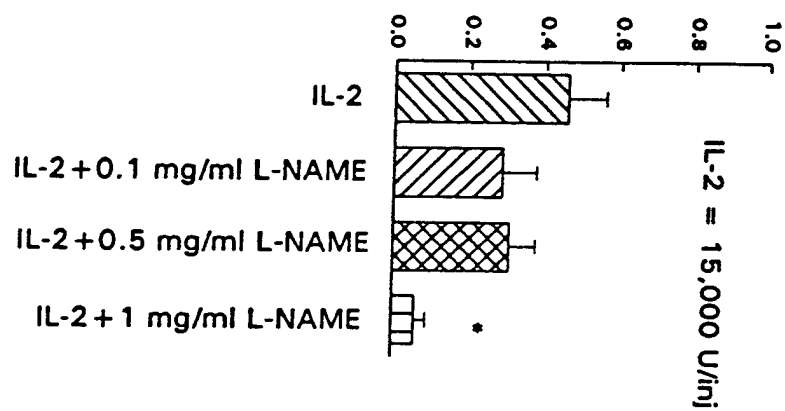
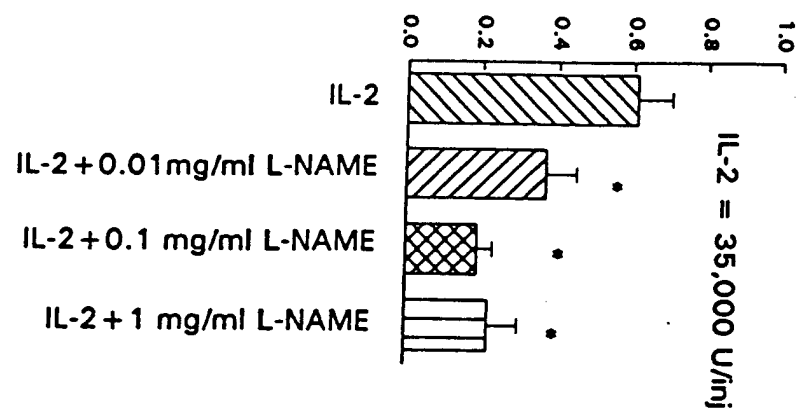
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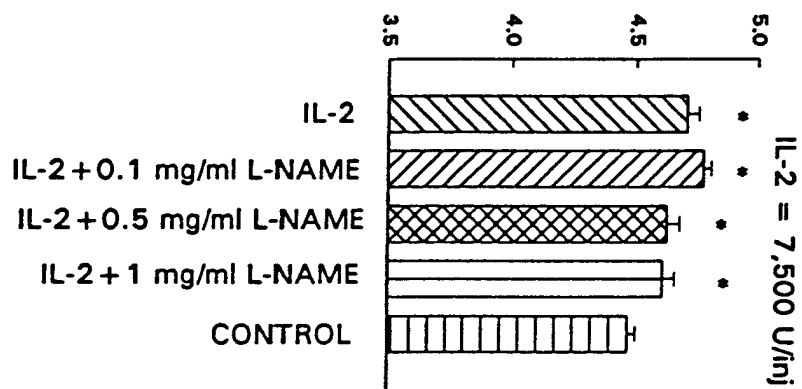
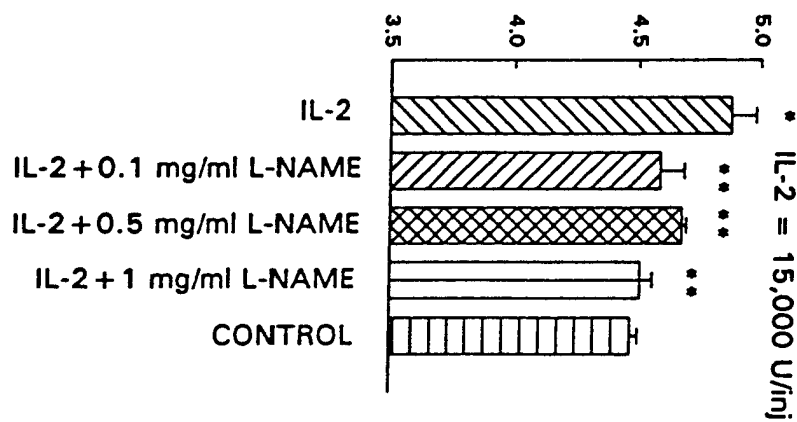
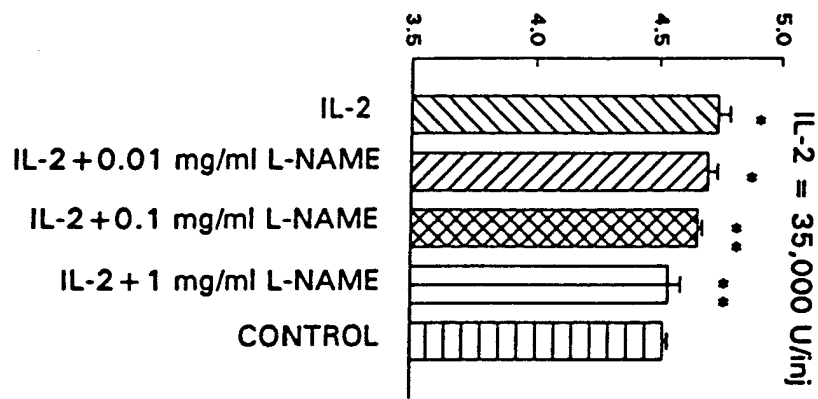
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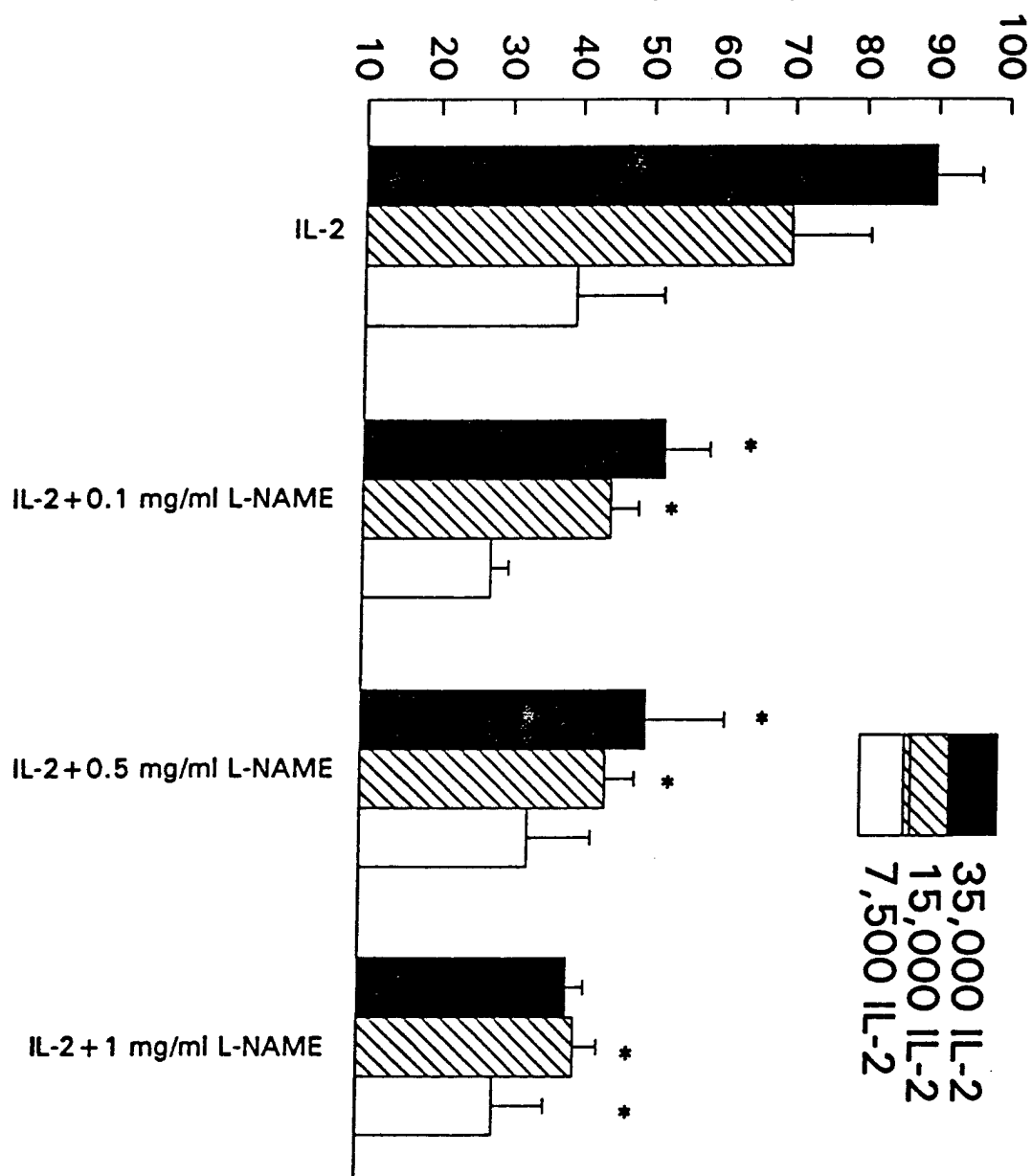
Volume of the pleural fluid (ml)

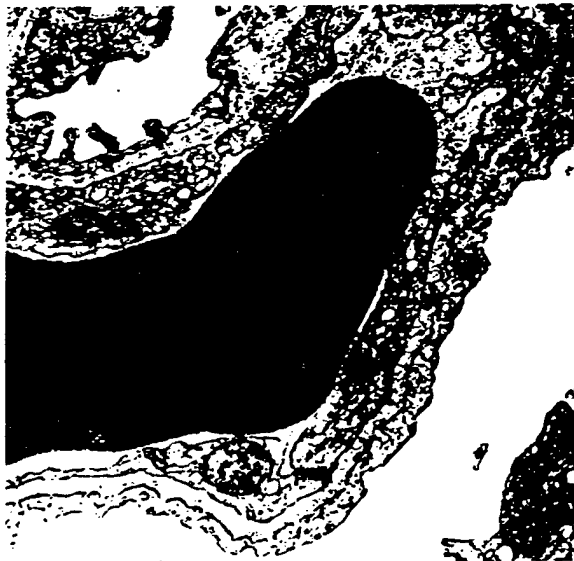
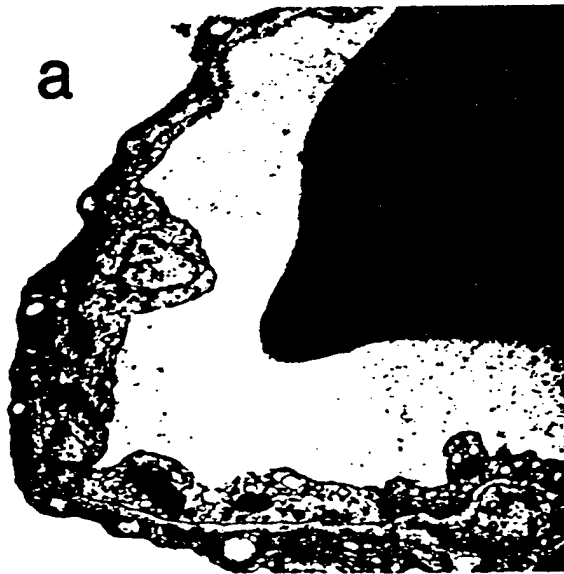


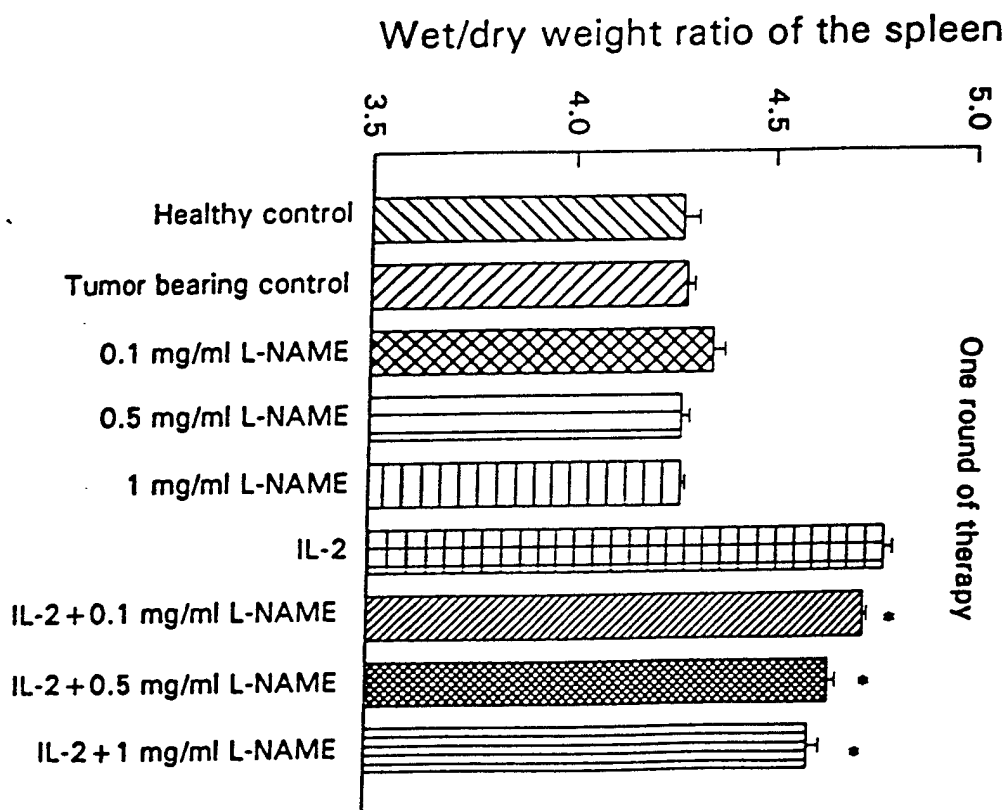
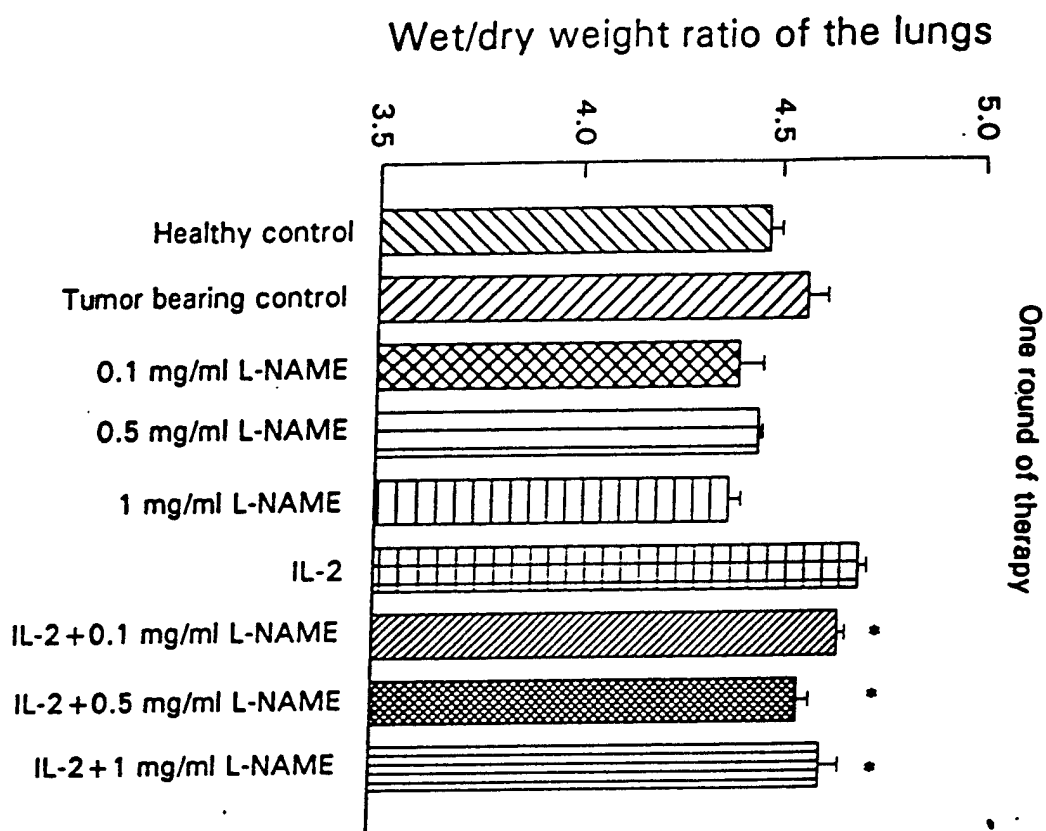
Wet/dry weight ratios of the lungs

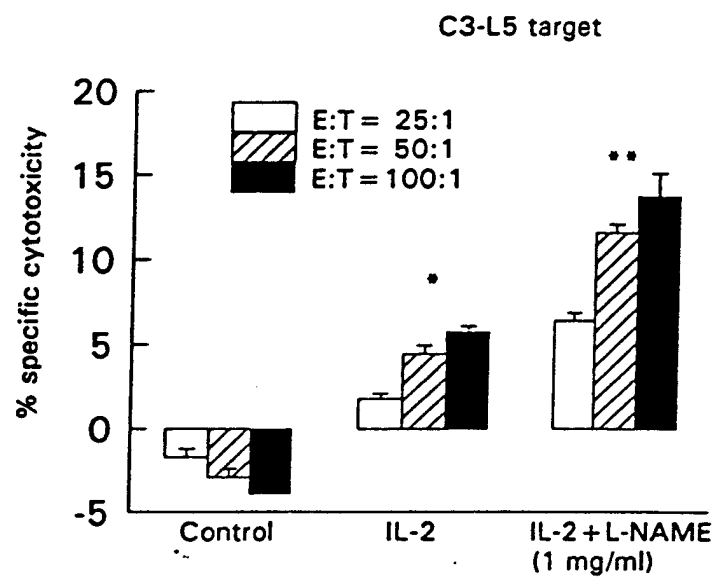
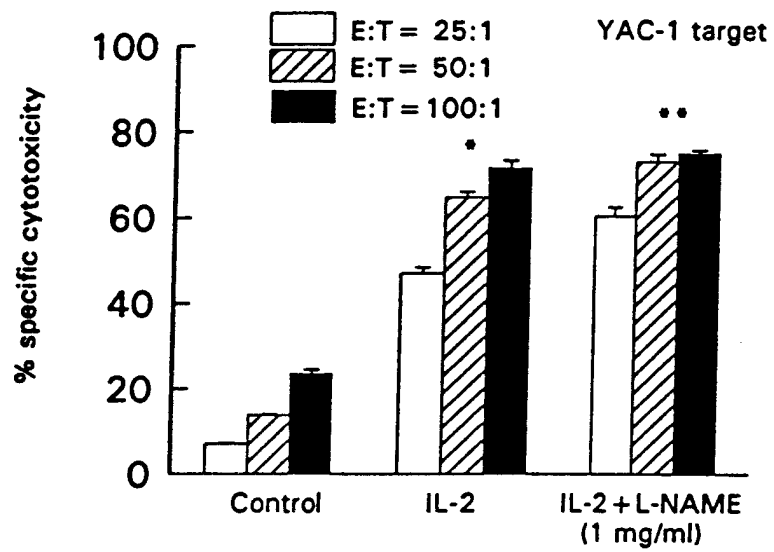


$\text{NO}_2^- + \text{NO}_3^-$  levels in the pleural fluid  
( $10^{-6}\text{M}$ )











# The Role of Active Inducible Nitric Oxide Synthase Expression in the Pathogenesis of Capillary Leak Syndrome Resulting from Interleukin-2 Therapy in Mice

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**SUMMARY:** Previously, we showed that nitric oxide (NO) plays a major role in the pathogenesis of IL-2-induced capillary leak syndrome in healthy or mammary adenocarcinoma-bearing C3H/HeJ mice. NO synthase (NOS) inhibitors, such as N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) reduced all the manifestations of IL-2-induced capillary leakage, without compromising the antitumor effects of IL-2. The present study was carried out on healthy C3H/HeJ mice subjected to one or two 4-day rounds of systemic IL-2 therapy with or without oral L-NAME therapy to: (a) identify the tissue source of NOS activity and NOS protein induced by IL-2 therapy; (b) identify histologically the nature of the structural damage to the lungs associated with IL-2 therapy-induced pulmonary edema; and (c) evaluate the effects of additional L-NAME therapy on the above-mentioned parameters. Results revealed that IL-2 therapy in healthy mice resulted in the expression of inducible NOS in numerous tissues including the endothelium and muscles of the anterior thoracic wall as well as splenic macrophages. One round of IL-2 therapy resulted in high levels of inducible NOS (iNOS) activity in the anterior thoracic wall accompanied by pleural effusion. After two rounds of IL-2 therapy, there was neither pleural effusion nor high iNOS activity in the thoracic wall. IL-2-induced pulmonary edema after one round of therapy correlated to both a significant rise in NO production measured in the serum and structural damage to the lungs and its capillaries. Addition of the NOS inhibitor L-NAME totally eradicated NOS activity but not necessarily iNOS expression. It also reduced IL-2-induced pulmonary edema and pleural effusion, restrained the rise in the levels of NO metabolites (nitrites and nitrates) in the serum and pleural effusion, and significantly restored the structural integrity of the lungs after one round of therapy. Thus, NOS inhibitors may be beneficial adjuncts to IL-2 therapy for cancer and infectious diseases. (*Lab Invest* 1997, 76:53-65).

IL-2 therapy alone or in combination with ex vivo-generated lymphokine-activated killer (LAK) cells (Rosenberg, 1989; Fisher et al, 1988; Dutcher et al, 1989) or immunomodulators, such as indomethacin (Mertens et al, 1993a, 1993b), has shown some promising results in treating patients with certain forms of

cancer, particularly melanomas and renal cell carcinomas. However, capillary leak syndrome (CLS), which is characterized by retention of extravascular fluid and severe hemodynamic instability inclusive of hypotension, remains a major roadblock to IL-2-based immunotherapy for cancer and infectious diseases (Siegel and Puri, 1991; Oppenheim and Lotze, 1994).

Excessive production of nitric oxide (NO), a short-lived biologic mediator (Palmer et al, 1987; Knowles and Moncada, 1994) synthesized—with the help of a family of enzymes called NO synthases (NOS)—by many mammalian cells from the amino acid L-arginine (Knowles and Moncada, 1994; Morris and Billiar, 1994), has been implicated in the pathogenesis of septic and endotoxic shock (Petros et al, 1991; Kilbourn et al, 1990; Wright et al, 1992). The high NO levels found in cancer patients receiving IL-2 therapy (Hibbs et al, 1992; Ochoa et al, 1992; Miles et al, 1994)

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seem to be responsible for severe hypotension, a common side effect of high-dose IL-2 therapy, because the condition is controllable with NOS inhibitors (Kilbourn et al, 1995; Fonseca et al, 1994).

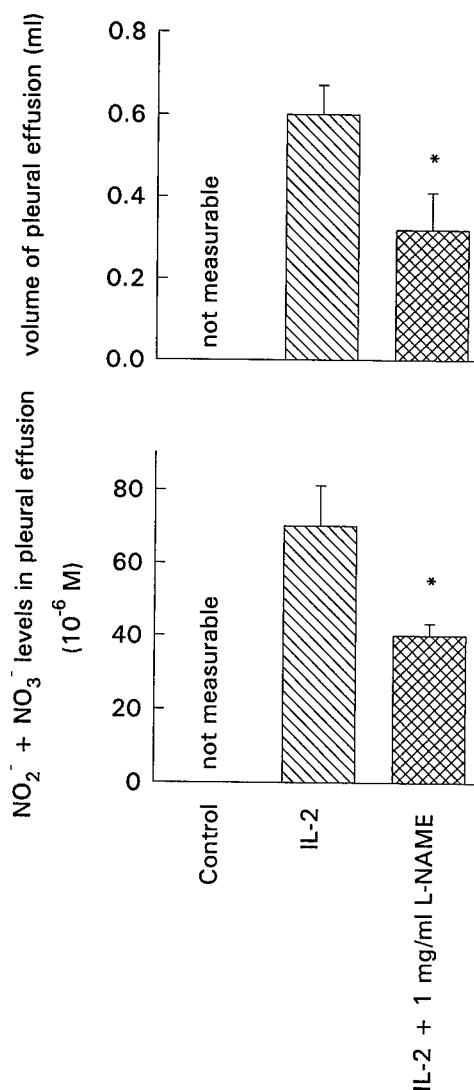
We previously demonstrated (Orucevic and Lala, 1996c, 1996d) that moderate to high doses of IL-2 therapy in healthy or mammary adenocarcinoma-bearing C3H/HeJ mice resulted in capillary leakage, as indicated by pleural effusion and fluid retention in the lungs, spleen, and kidneys. We also showed that stable metabolic products of NO measured in the pleural effusion and the serum of these animals were directly related to the IL-2 dose. It was also shown that therapy with L-NAME, an NOS inhibitor administered in the animals' drinking water, significantly mitigated all of the manifestations of IL-2-induced CLS and led to a concomitant reduction of IL-2-induced NO production in the serum and pleural effusion. These results, combined with our previous findings of mitigation of IL-2-induced capillary leakage with oral  $N^G$ -methyl-L-arginine (NMMA), another NOS inhibitor (Orucevic and Lala, 1996a), strongly indicate that NO plays a major role in the pathogenesis of CLS induced by high doses of IL-2.

We hypothesize that IL-2 therapy directly or indirectly causes inducible NOS (iNOS) protein and iNOS activity in specific cells, causing an increase of NO production in local tissues, which leads, in turn, to CLS through the dual mechanism of NO-induced damage to endothelial cells (Palmer et al, 1992; Estrada et al, 1992) and vasodilation (Palmer et al, 1987). Whereas the former caused a direct leakage of the capillaries, the latter also resulted in systemic hypotension, which then indirectly caused pulmonary hypertension, thus further precipitating the pulmonary edema. Both mechanisms may also underlie the IL-2-induced pleural effusion in the mouse inasmuch as the blood in the murine pleura is partially derived from the pulmonary arteries (Pinchon et al, 1980). The present study was therefore designed in healthy C3H/HeJ mice to: (a) identify the tissue source of NOS activity and NOS protein induced by IL-2 therapy; (b) histologically identify the nature of structural damage to the lungs during IL-2 therapy-induced pulmonary edema; and (c) test whether the addition of L-NAME therapy abrogated the increase in NOS activity and IL-2-induced structural damage to the lungs. NOS activity of the lungs and the anterior thoracic wall, iNOS expression in the lungs, anterior thoracic wall, and spleen, as well as morphologic changes in the lungs were all evaluated in mice subjected to systemic IL-2 therapy with or without oral L-NAME therapy.

## Results

### **Effects of L-NAME on IL-2-Induced Pleural Effusion and $NO_2^- + NO_3^-$ Levels in the Serum and Pleural Effusion after the First Round of Therapy**

As we have previously reported in other studies (Orucevic and Lala, 1996d), L-NAME significantly reduced pleural effusion induced by IL-2 after the first round of therapy (Fig. 1, top). We also confirmed that IL-2-induced increases in  $NO_2^- + NO_3^-$  levels in the pleural effusion are significantly reduced by addition of L-NAME (Fig. 1, bottom), a finding similar to those reported in our earlier studies (Orucevic and Lala, 1996d).



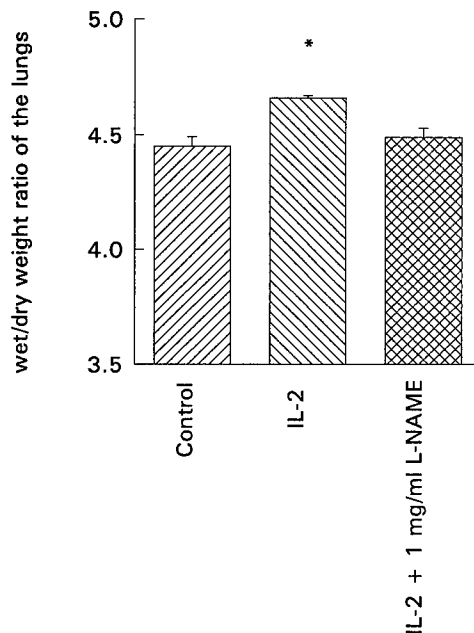
**Figure 1.**

Pleural effusion (top) and  $NO_2^- + NO_3^-$  levels in the pleural effusion (bottom) after IL-2 and IL-2 + L-NAME therapy. Data represent mean  $\pm$  SE ( $n = 5$ ); \* indicates significant difference from IL-2 treatment ( $p < 0.05$ ). L-NAME therapy (1 mg/ml of drinking water) significantly ( $p < 0.05$ ) reduced IL-2 (15,000 U/mj) induced pleural effusion and IL-2-induced rise in nitrite and nitrate levels in the pleural effusion. Control (untreated) mice did not show any pleural effusion.

### Effects of L-NAME on IL-2-Induced Morphologic Changes of the Lungs after the First Round of Therapy

Light microscopic image analysis (Mocha Image Software; Jandel Scientific, San Rafael, California) of semithin sections of the lungs (Fig. 3) revealed that, in comparison with the control lungs (Fig. 3 and Table 1), IL-2 therapy led to a significant increase in the area occupied by the connective tissue in the lung and, conversely, a significant reduction in the area occupied by air spaces as a result of IL-2-induced pulmonary edema (Fig. 2). The addition of L-NAME therapy, however, significantly reduced IL-2-induced pulmonary edema (Fig. 2) and significantly restored the balance between the area occupied by the connective tissue and air spaces to close to control levels (Fig. 3 and Table 1).

Ultrastructural analysis of the lungs (Fig. 4) revealed that IL-2 therapy led to major distortions in the capillary ultrastructure of the lungs. The changes included swelling of endothelial cells and type I pneumocytes, thickening of the basement membrane of the thin portion of the capillaries, or herniation of the endothelial cell into the vessel lumen because of accumulation of fluid between the plasma membrane and the basement membrane, and the presence of cellular debris in the blebs in endothelial cells or type I pneumocytes, indicating cellular damage (Fig. 4). Addition of L-NAME therapy reduced the ultrastructural damage



**Figure 2.**

Water content in the lungs after one round of IL-2 and IL-2 + L-NAME therapy. Data represent mean ± SE ( $n = 5$ ). \* indicates significant difference from control ( $p < 0.05$ ). Addition of L-NAME (1 mg/ml of drinking water) significantly ( $p < 0.05$ ) reduced IL-2 (15,000 U/inj) induced pulmonary edema.

**Table 1. Percentage of Area Occupied by the Connective Tissue in the Lungs<sup>a</sup> after IL-2 and L-NAME therapy, expressed as mean ± SE ( $n = 5$ ).**

Treatment	% of Connective Tissue Area
IL-2	69 ± 2.5 <sup>b</sup>
IL-2 + L-NAME	62.4 ± 1.3 <sup>c</sup>
Control	55.2 ± 3.1

<sup>a</sup> Light microscopic image analysis (Jandel Scientific Mocha image software) of semithin sections of the lungs from animals treated with IL-2 (15,000 CU/inj., 10 inj., i.p. every 8 hours) or IL-2 + L-NAME (1 mg/ml of drinking water, starting 1 day before IL-2 therapy); <sup>b</sup> significant ( $p < 0.05$ ) increase in the relative area occupied by the connective tissue, compared to control; <sup>c</sup> significant ( $p < 0.05$ ) reduction in the relative area occupied by the connective tissue, compared to IL-2.

induced by IL-2. Although some swelling of endothelial cells remained, there was no noticeable damage of endothelial cell membranes or thickening of the basement membrane of the thin portion of the capillaries (Fig. 4).

### Effects of L-NAME on IL-2-Induced Capillary Leakage and NO Production after Two Rounds of Therapy

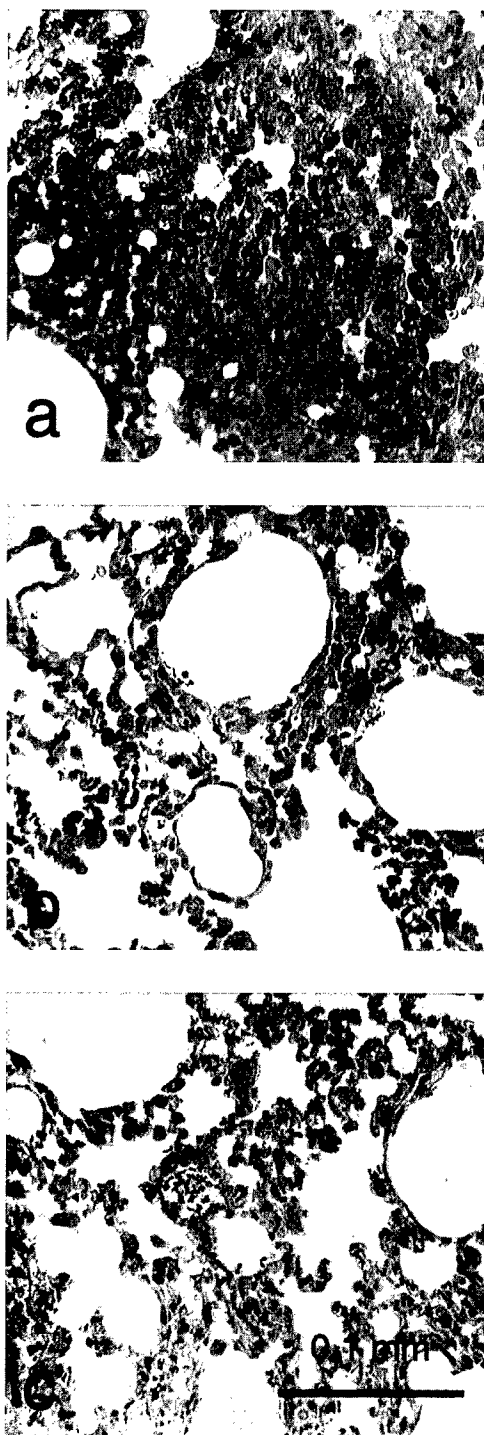
IL-2 therapy caused an increase in the water content in the lungs after the second round of therapy, however, this was not significantly affected by the addition of L-NAME therapy (Fig. 5). Similarly, L-NAME therapy at this point was not effective in causing a significant reduction in the  $\text{NO}_2^- + \text{NO}_3^-$  levels in the serum (data not presented). There was no pleural effusion at this time in animals treated with either IL-2 alone or IL-2 with L-NAME, as had been documented earlier (Orulevic and Lala, 1996c, 1996d).

### NOS Activity in the Lungs and Anterior Thoracic Wall after One or Two Rounds of IL-2 and L-NAME Therapy

One round of IL-2 therapy significantly increased  $\text{Ca}^{2+}$ -independent (primarily explained by inducible) NOS activity in the anterior thoracic wall (Fig. 6), whereas the increase in  $\text{Ca}^{2+}$ -independent NOS activity in the lungs was not significant (Fig. 7). The second round of IL-2 therapy, however, increased  $\text{Ca}^{2+}$ -independent NOS activity in the lungs but did not induce any  $\text{Ca}^{2+}$ -independent NOS activity in the thoracic wall (Figs. 6 and 7). The addition of L-NAME therapy during the first or the second round of IL-2 therapy eradicated NOS activity in both the lungs and anterior thoracic wall (Figs. 6 and 7).

### Tissue Distribution of Immunoreactive iNOS Protein During IL-2 with or without L-NAME Therapy

**Thoracic Wall.** Immunocytochemical staining for iNOS enzyme revealed that one round of IL-2 therapy



**Figure 3.**

Effects of IL-2  $\pm$  L-NAME therapy on histology of the lungs. a, IL-2; b, IL-2 + L-NAME; c, control; semithin sections of the lungs stained with toluidine blue. Sections of the lungs from IL-2-treated animals showed a significant increase in the area occupied by the connective tissue of the lungs as well as interstitial mononuclear cell infiltration (a). The addition of L-NAME therapy significantly reduced IL-2-induced pulmonary edema and mononuclear cell infiltration and restored the balance between the area occupied by the connective tissue and air spaces (IL-2 + L-NAME versus control).

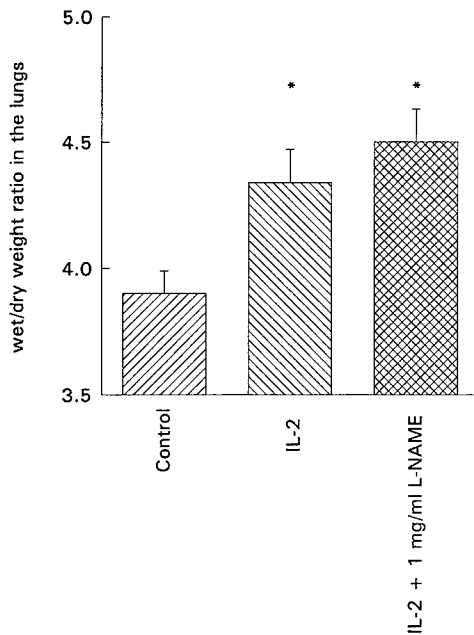
induced iNOS expression in endothelial cells of capillaries surrounding the fibers of intercostal muscles of the anterior thoracic wall (Fig. 8b). The addition of L-NAME therapy, although eradicating all NOS activity



**Figure 4.**

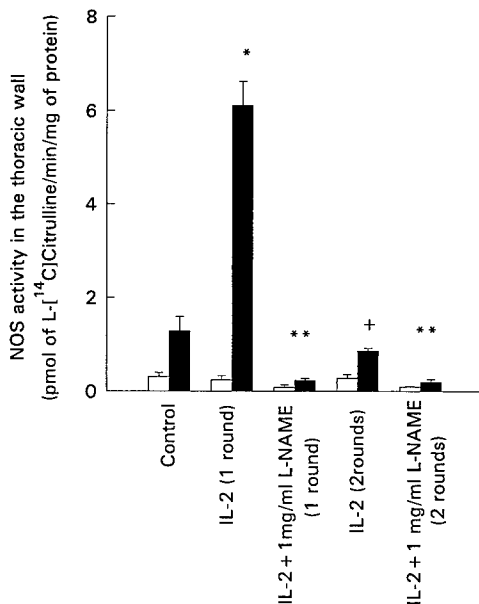
Ultrastructure of the lungs of mice given IL-2 or IL-2 + L-NAME therapy. a, control; b, IL-2; c, IL-2 + L-NAME; magnification,  $\times 17,120$ . Basement membrane is thick ( $\leftarrow$ ) and discontinuous in IL-2-treated mice. Endothelial as well as pneumocyte type I cells are severely damaged. There is also swelling of endothelial cells as well as pneumocyte type I.  $\leftarrow$  indicates an area of blood-air barrier showing such damage. Basement membrane is continuous and thin at the thin part of the capillary (\*) in IL-2 + L-NAME-treated animals. Endothelial cells, although in some cases remaining swollen, are never detached from their basement membrane in these mice.

from the anterior thoracic wall (Fig. 6), did not influence the expression or distribution of iNOS enzyme. After two rounds of IL-2 with or without L-NAME, there



**Figure 5.**

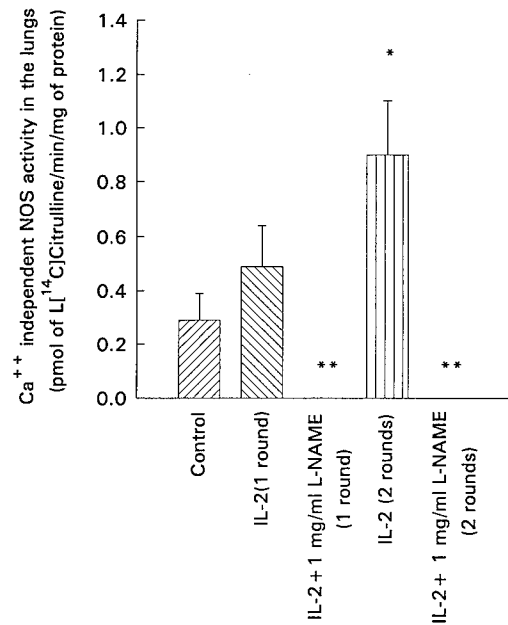
Water content of the lungs after the second round of IL-2 ± L-NAME therapy. Data represent mean ± SE ( $n = 5$ ). \* indicates significant difference from control ( $p < 0.05$ ). Addition of L-NAME therapy did not influence IL-2-induced pulmonary edema after the second round of therapies.



**Figure 6.**

NOS activity in the thoracic wall after one or two rounds of IL-2 ± L-NAME therapy. \*, one round of IL-2 therapy significantly ( $p < 0.05$ ) increased  $\text{Ca}^{2+}$ -independent NOS activity in the anterior thoracic wall. \*\*, addition of L-NAME therapy abolished both forms of NOS activity, after either one or two rounds of therapy. +, there was no significant iNOS activity induced by the second round of IL-2 therapy. □,  $\text{Ca}^{2+}$ -dependent activity; ■,  $\text{Ca}^{2+}$ -independent activity.

was stronger staining for iNOS enzyme present in the endothelial cells of capillaries surrounding the fibers of intercostal muscles, as well as a punctate staining of some muscle fibers (Fig. 8, d and e). Electron micro-



**Figure 7.**

$\text{Ca}^{2+}$ -independent iNOS activity in the lungs after one or two rounds of IL-2 ± L-NAME therapy. \*, IL-2 therapy induced significant ( $p < 0.05$ ) iNOS activity in the lungs after the second round of therapy. \*\*, L-NAME therapy abolished all iNOS activity in the lungs after either one or two rounds of therapy.

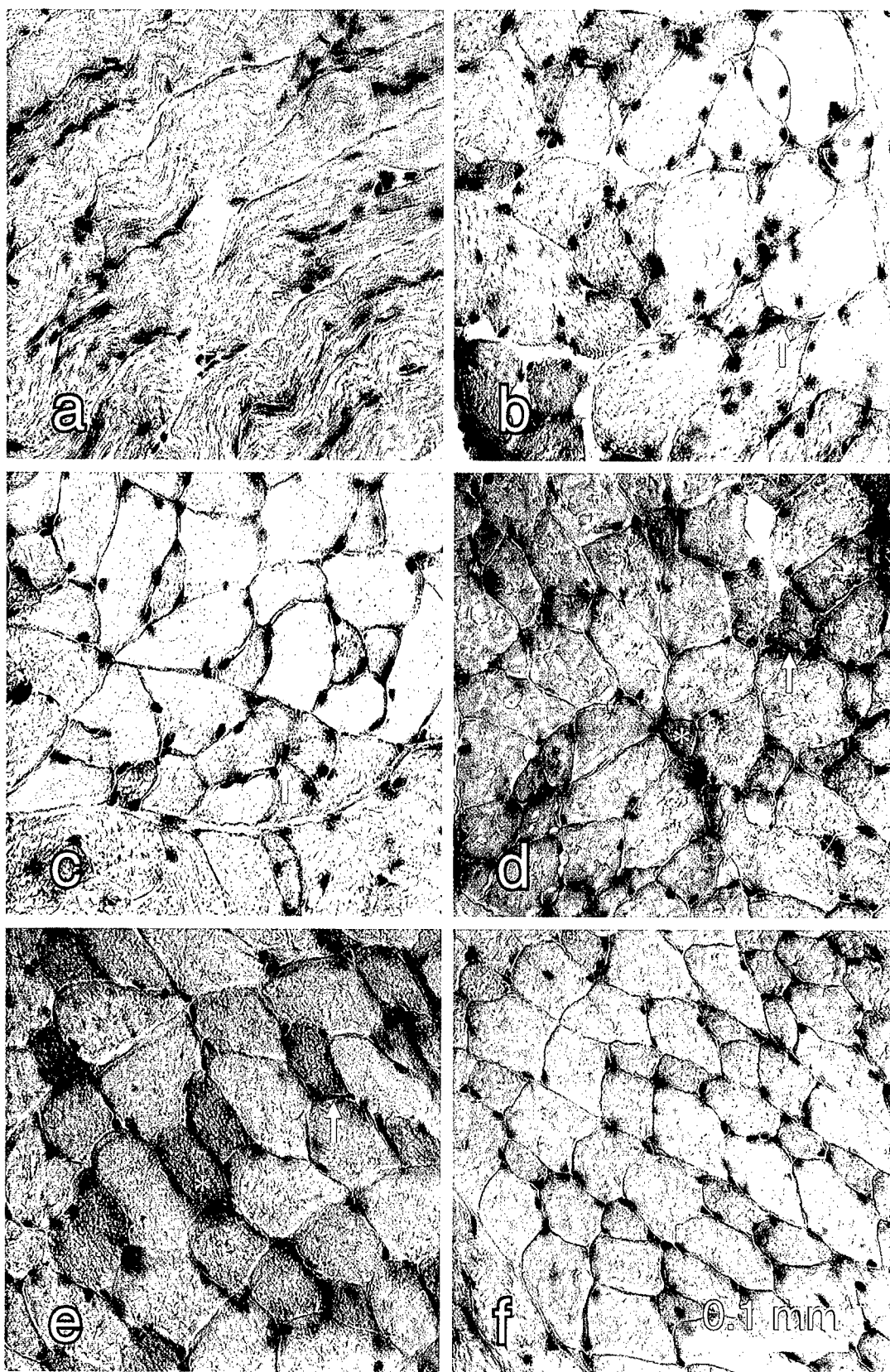
scopic immunocytochemistry revealed that iNOS enzyme was present in the sarcoplasm of some intercostal muscles (Fig. 9a) and in the cytoplasm of endothelial cells of capillaries and small arteries (Fig. 9b).

**Lungs.** Although NOS activity in the lungs was significantly induced by two rounds of IL-2 therapy (Fig. 7), there was no significant difference in iNOS staining between the lungs of control and treated mice (data not presented).

**Spleen.** iNOS-positive macrophages were scarce in the spleens of normal mice but abundant in the spleens of IL-2 or IL-2 + L-NAME-treated animals after one or two rounds of therapy (Fig. 10). After one round of therapy, iNOS positive macrophages were present mostly in the red pulp of the spleens of IL-2-treated mice, whereas in mice subjected to IL-2 + L-NAME, they had accumulated at the periphery of the white pulp. After two rounds of therapy, iNOS-positive macrophages accumulated within the white pulp of IL-2-treated animals and were most numerous at the periphery of the white pulp of IL-2 + L-NAME-treated animals.

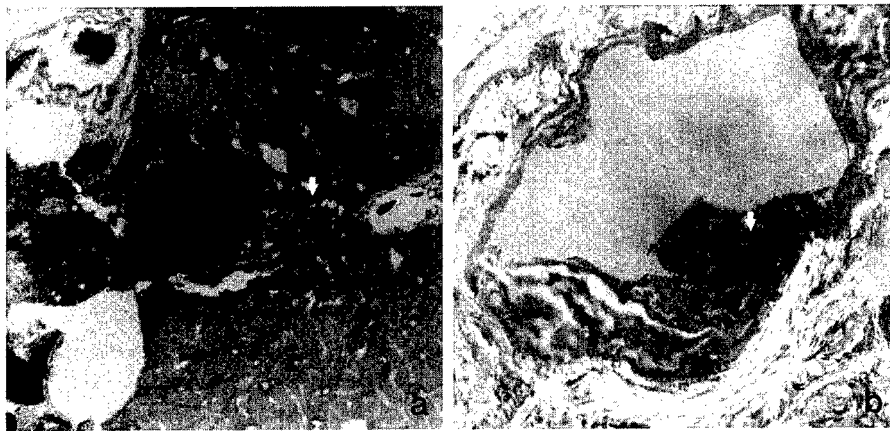
## Discussion

The present study revealed that IL-2 therapy in healthy mice induced significant  $\text{Ca}^{2+}$ -independent (ie, inducible) NOS activity in the lungs and the thoracic wall concomitant with the induction of pulmonary edema



**Figure 8.**

Immunostaining with a mouse monoclonal antibody against iNOS (a to e), and a negative control antibody of the same isotype (f) in the anterior thoracic wall after one or two rounds of IL-2  $\pm$  L-NAME therapy, lightly counterstained with hematoxylin. a, control mice; b, IL-2, 1 round; c, IL-2 + L-NAME, 1 round; d, IL-2, 2 rounds; e and f, IL-2 + L-NAME, 2 rounds. Strong endothelial cell staining ( $\uparrow$ ) is present in all IL-2-treated groups. Staining of endothelial cells appears stronger after two rounds of therapy, when staining is also noted in some muscle fibers (\*). There was no positive immunostaining in the presence of a negative control antibody of the same isotype in any tissue of the anterior thoracic wall after one or two rounds of IL-2  $\pm$  L-NAME therapy (f).



**Figure 9.**

Electron microscopic immunolocalization of iNOS protein in the intercostal muscle fibers (a) and arteriole (b) in the anterior thoracic wall from a mouse treated with two rounds of IL-2. iNOS immunoreactivity was located in the sarcoplasm of some intercostal muscles (a) as well as in the cytoplasm of endothelial cells (b) of arterioles. Magnification,  $\times 5715$ .

and pleural effusion. The addition of oral L-NAME therapy ameliorated these more severe symptoms of IL-2-induced capillary leakage and coincided with the abrogation of NOS activity in these tissues, suggesting that a high local NOS activity was instrumental in the pathogenesis of capillary leakage.

Pleural effusion after one round of IL-2 therapy was accompanied by an induction of highly active NOS enzyme in the anterior thoracic wall. The enzyme was mostly localized in the endothelium of the capillaries surrounding the intercostal muscle fibers. Because the parietal pleura is supplied by the vessels that also supply the thoracic wall, it is likely that this local source of NO contributed to the high NO level in the pleural fluid. There was no pleural effusion in mice after two rounds of IL-2 therapy, confirming our previously reported findings (Orulevic and Lala, 1996c, 1996d). Interestingly, there was also a lack of significant induction of iNOS activity in the thoracic wall at this time, which reconfirms the association of pleural effusion with local NOS activity. The findings of low NOS activity can, perhaps, best be explained by the feedback inhibition of NOS activity by high NO levels, as reported by Moncada's group for a macrophage cell line *in vitro* (Assreuy et al, 1993). Subsequently, it was demonstrated by Luss et al (1994) that NO not only exerted feedback inhibition of NOS activity but also decreased the level of iNOS protein expression, most likely by inhibiting translation of the iNOS protein. Surprisingly, however, an abundance of immunoreactive iNOS protein (both after the second round of IL-2 or IL-2 + L-NAME) was still detected in the anterior thoracic wall in the present study. Electron microscopic immunocytochemistry confirmed that the protein was present within the endothelium of capillaries surrounding the intercostal muscle fibers and

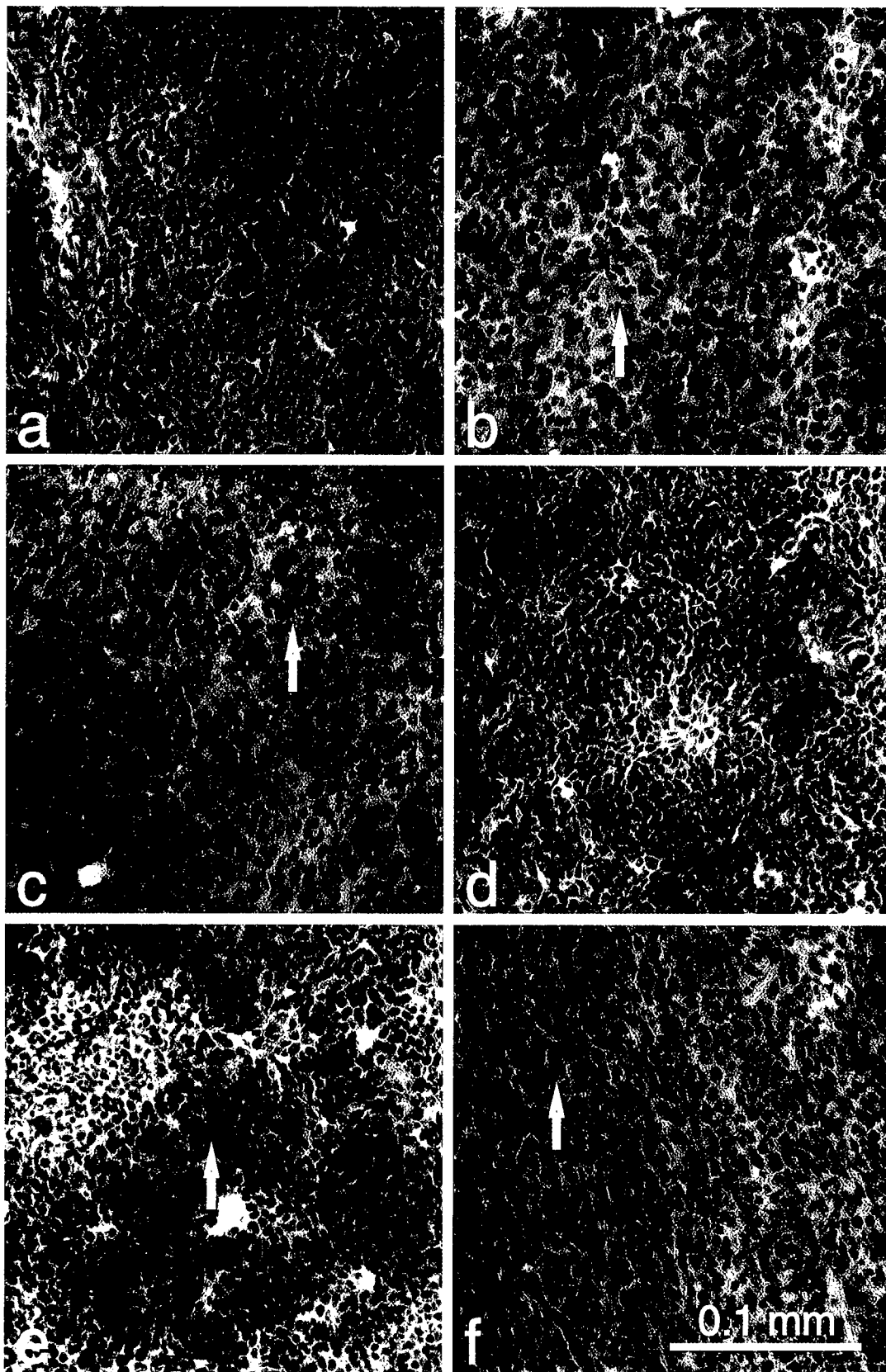
also some muscle fibers. Currently, it is not possible to give an explanation for the detection of iNOS protein in the absence of iNOS activity after the second round of IL-2. This paradox remains to be resolved by further quantitative studies, eg Western blot analysis of the iNOS protein.

IL-2-induced pulmonary edema was present after both rounds of IL-2 therapy and was accompanied by a significant NOS activity in the lungs. Addition of L-NAME therapy during either round abolished NOS activity but reduced pulmonary edema only after first round of IL-2 therapy. Thus, one would assume that NO induction as well as other mechanisms may be responsible for the presence of pulmonary edema after the second round of IL-2 therapy, so that inhibition of NO synthesis with L-NAME was not enough to prevent IL-2-induced pulmonary edema. It is likely that a direct LAK-cell-mediated injury to the pulmonary endothelium was greater after the second round of IL-2.

Although L-NAME was effective in abolishing the NOS activity in the lungs and the thoracic wall after the second round of IL-2 therapy, the reduction in serum NO level was not significant. This may indicate that L-NAME did not totally eradicate iNOS activity in all cells that contributed to the serum NO level after the second round of therapy. It remains to be investigated whether more selective iNOS inhibitors would provide a better therapeutic efficacy against IL-2-induced capillary leakage.

A significant increase in the thickness of the inter-alveolar connective tissue space (as measured by computerized histomorphometry) accompanied IL-2-induced pulmonary edema. Histologic analysis revealed marked lymphocyte infiltration similar to those showed by Parhar and Lala (1987) and Dubinett et al





**Figure 10.**

Immunostaining with a mouse monoclonal antibody against iNOS (a to c, e and f) and a negative control antibody of the same isotype (d) in the spleen after one or two rounds of IL-2  $\pm$  L-NAME therapy, counterstained with hematoxylin. a, control; b, IL-2, 1 round; c and d, IL-2 + L-NAME, 1 round; e, IL-2, 2 rounds; f, IL-2 + L-NAME, 2 rounds. ( $\uparrow$ ) Staining is present in a substantial population of cells (identified as macrophages at higher magnifications) in the spleens of all IL-2-treated groups. There was no positive immunostaining in the presence of a negative control antibody of the same isotype in the spleen after one or two rounds of IL-2  $\pm$  L-NAME therapy (d).



(1994). Ultrastructural analysis revealed that IL-2-induced pulmonary edema was associated with a major distortion in the capillary architecture of the lungs. The changes included swelling or damage of endothelial cells and type I pneumocytes, thickening of the basement membrane of the thin portion of the capillaries, or herniation of the thin segment of endothelial cells into the vessel lumen because of the accumulation of fluid between the plasma membrane and the basement membrane. Similar findings were described in the lungs of rats and rabbits treated with IL-2 (Renzi et al, 1991; Goldblum et al, 1990). Dubinett et al (1994) suggested that IL-2-induced tumor necrosis factor (TNF)  $\alpha$  played a central role in mediating the pulmonary vascular leakage, because IL-2 upregulated the in situ expression of both the TNF $\alpha$  mRNA and the protein in the lungs, whereas administration of a soluble TNF $\alpha$  receptor significantly reduced IL-2-induced pulmonary edema. The present study revealed that NO inhibition with L-NAME reduced IL-2-induced pulmonary edema, concomitant with a significant reduction of the thickness of interalveolar spaces. This was associated with ultrastructural evidence of significant restoration of capillary architecture in the lungs. Because TNF $\alpha$  caused endothelial cell damage in vitro through the production of NO (Palmer et al, 1992; Estrada et al, 1992), it is reasonable to postulate that IL-2-induced TNF $\alpha$  production in vivo may have been instrumental in high NO production, leading to pulmonary edema.

Macrophages immunoreactive for the iNOS protein became abundant in the spleen of IL-2- or IL-2 + L-NAME-treated animals after either one or two rounds of therapies. It appeared that there were more macrophages stained in the spleens of mice treated with IL-2 + L-NAME, than with IL-2 alone. Luss et al (1994) reported that chronic inhibition of NO production can result in an increase in iNOS mRNA and protein levels in iNOS expressing cells, although such iNOS may remain inactive because of the presence of L-NAME. We have earlier shown (Orucevic and Lala, 1996b) that in vivo LAK-cell activation in splenocytes of IL-2 + L-NAME-treated mice was significantly higher than that in IL-2-treated animals, suggesting that the IL-2-induced increase in NOS activity followed by increased NO production within the spleen interfered with optimal LAK cell activation. Whether a cessation of L-NAME therapy would lead to a rebound in NO production in the presence of high iNOS protein levels and whether this would influence splenocyte cytotoxicity remains to be investigated.

In conclusion, the present study revealed that a rise in NO metabolites in the tissue fluids was associated

with a high local NOS activity as well as expression of iNOS protein during IL-2 therapy. A reduction of NOS activity, but not necessarily the expression of iNOS protein, was a good indicator of therapeutic effectiveness in the amelioration of capillary leakage with L-NAME therapy. It is likely that NOS inhibitors can be useful adjuncts to IL-2 therapy of cancer and infectious diseases.

## Materials and Methods

### Mice

C3H/HeJ female mice (7 to 8 weeks old) were obtained from the Jackson Laboratories (Bar Harbor, Maine). Animals were fed standard mouse chow, provided with water ad libitum, and kept on a 12-hour light/dark cycle. Animal care was in accord with the guidelines set out by the Canadian Council on Animal Care.

### Interleukin-2

Recombinant, highly purified human IL-2 (lot LQP-046) was kindly provided by the Chiron Corporation (Emeryville, California). The specific activity was  $3 \times 10^6$  Cetus Units (CU) or  $18 \times 10^6$  IU/mg of IL-2. The lyophilized IL-2 (1.2 mg/vial) was first reconstituted with 1 ml of distilled water. RPMI-1640 medium (ICN Biomedicals, Inc., Costa Mesa, California) was used to dilute it further to obtain 15,000 CU in 0.1 ml per injection. Previously, we found this dose to induce capillary leakage (Orucevic and Lala, 1996a, 1996d). The reconstituted material was stored at 4°C up to 1 day.

### L-NAME

L-NAME obtained from Sigma Chemical Company (St. Louis, Missouri) was added to the animals' drinking water to provide concentrations of 1 mg/ml. We previously found this dose to significantly reduce IL-2-induced capillary leakage and to increase IL-2-induced NO production in healthy mice (Orucevic and Lala, 1996d).

### Experimental Design

Healthy mice ( $n = 20$  per group) received one of the following treatments: nothing; 15,000 CU of IL-2 alone given ip every 8 hours for 10 injections; or IL-2 + L-NAME (1 mg/ml of drinking water starting 1 day before IL-2 therapy). Mice ( $n = 8$  per group) were killed 1 hour after the last IL-2 injection to measure NOS activity in the lungs and anterior thoracic wall, as well as tissue distribution of iNOS enzyme in the lung,

intercostal muscles, and spleen. Water content in the pleural cavities (known to be a reliable marker of IL-2-induced CLS in mice; Orucevic and Lala, 1996a, 1996d) and  $\text{NO}_2^- + \text{NO}_3^-$  levels in the serum and pleural effusion were also measured. Structural changes of the lungs during IL-2 therapy were examined using light and electron microscopy. All of the above parameters were examined to establish the relationship between NOS activity and protein expression within the tissues and the degree of IL-2-induced CLS.

The remainder of the mice ( $n = 12$  per group) were given a second round of IL-2, which started 6 days after the first round and followed the same schedule, doses, and route of administration as the first round. L-NAME was also given in drinking water (1 mg/ml, starting 1 day before the second round of IL-2). Mice were killed at the end of the second round of IL-2 (1 hour after the last IL-2 injection) to measure the same parameters as the first round. The differences in NOS activity or iNOS protein expression between one and two rounds of IL-2 therapy were then examined.

#### **Measurement of Pleural Effusion, Pulmonary Edema, and NO Production**

For measurement of water content, the left lung was recovered from animals, and its wet weight was recorded. The lungs were frozen at  $-80^\circ\text{C}$  and then freeze dried to constant weight in a freeze-drying system (Labconco Corporation, Kansas City, Missouri). Dry weights were measured, and wet to dry weight ratio of the lungs was calculated (Orucevic and Lala, 1996c, 1996d).

The volume of liquid from both pleural cavities was measured directly by complete aspiration with a 1-ml syringe (Orucevic and Lala, 1996c, 1996d).

Samples of serum and pleural effusion were collected after the end of treatments to measure  $\text{NO}_2^-$  and  $\text{NO}_3^-$ , the principal metabolites of NO (Moncada and Higgs, 1993; Kelm et al, 1992). Cadmium filings were used for the conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  (Davison and Woof, 1978); Griess reaction was used to measure  $\text{NO}_2^-$  (Green et al, 1982). The basal concentrations of  $\text{NO}_2^-$  were not detectable; we were, however, able to obtain measurable amounts of  $\text{NO}_2^-$  from our samples by reducing the  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . The absorbance was read at 543 nm in a DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, California). The final concentration of nitrite in pleural effusion and serum was calculated from a sodium nitrite standard curve, which was linear from 0 to 90  $\mu\text{M}$  nitrite (Orucevic and Lala, 1996c, 1996d).

#### **Light and Electron Microscopy of the Lungs**

Lungs were fixed in 2.5% glutaraldehyde in 0.1 M/l of sodium cacodylate buffer by immersion, postfixed in 1% osmium tetroxide in 0.1 M/l cacodylate buffer, infiltrated, and embedded in plastic (epoxy resin). Semithin sections (0.5  $\mu\text{m}$ ) were stained with toluidine blue for light microscopic analysis. Thin sections (90 nm) were stained with lead citrate and uranyl acetate (Hunter, 1993) and examined with an electron microscope. Light microscopic image analysis (Jandel Scientific) was used to establish the degree of pulmonary edema (induced by IL-2 therapy and abrogated by L-NAME therapy) and to calculate the percentage of area occupied by the connective tissue of the lungs versus the percentage of area occupied by the air spaces of the lungs. Ultrastructural changes in the lung morphology (interalveolar septa as well as blood-air barrier) were analyzed through the use of electron microscopy. Alterations in endothelial and epithelial cell morphology, endothelial continuity, thickness of the endothelial and epithelial basement membranes, and the characteristics of migratory cells in the interalveolar septa were analyzed.

#### **Measurement of NOS Activity**

The lung and anterior thoracic wall (all tissues except the skin) were collected from mice receiving nothing or one or two rounds of IL-2  $\pm$  L-NAME, snap-frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$  until assayed for NOS activity. Assay of NOS was performed as described by Thomsen et al (1995). All reagents were obtained from Sigma unless otherwise stated.

Frozen tissue ( $n = 5$  per treatment group) was homogenized (with a polytron) in 5 volumes of a buffer containing 20 mM HEPES, 0.1 mM EDTA, 0.2 mM sucrose, 5 mM DL-dithiothreitol (Boehringer Mannheim, Laval, Quebec), 10  $\mu\text{g}/\text{ml}$  each of leupeptin (Boehringer Mannheim) and soybean trypsin inhibitor, and 1  $\mu\text{g}/\text{ml}$  pepstatin. The homogenates were then centrifuged at 10,000g at  $4^\circ\text{C}$  for 30 minutes. Endogenous arginine from obtained supernatants (cytosol plus microsomes) was removed by addition of 1:5 ratio of cation:exchange resin (Dowex 50 $\times$ 8 to 400), followed by short centrifugation (1 minute, 10,000g). NOS in the supernatants was measured by conversion of L-[U- $^{14}\text{C}$ ] arginine (Amersham Life Science, Clearbrook, Illinois) to [U- $^{14}\text{C}$ ] citrulline at  $37^\circ\text{C}$  for 10 minutes, as described by Salter et al (1991). In brief, 100  $\mu\text{l}$  of substrate (uninhibited substrate) containing 10  $\mu\text{M}$  tetrahydrobiopterin, 2.5 mM of DL-dithiothreitol, 4000 U/ml calmodulin, 250  $\mu\text{M}$   $\text{CaCl}_2$ , 0.5 mg/ml BSA, 125  $\mu\text{M}$  NADPH, 1500 pmol arginine (cold and hot),

and 100  $\mu\text{M}$  of L-Citrulline in HEPES buffer was incubated with 50  $\mu\text{l}$  of enzyme (supernatant) for 10 minutes in a water bath set at 37°C. The reaction was stopped with the addition of 500  $\mu\text{l}$  of Dowex and 1000  $\mu\text{l}$  of distilled water. Twenty to thirty minutes later, when Dowex had settled at the bottom of the tube, and theoretically all of the  $^{14}\text{C}$ -L-arginine had adhered to Dowex, 975  $\mu\text{l}$  of clear upper phase containing only  $^{14}\text{C}$ -L-citrulline was mixed with 3 ml of scintillation cocktail (Amersham Canada Ltd., Oakville, Ontario, Canada) and counted on the scintillation counter.

NOS activity in the citrulline assay was calculated from the difference of counts per minute between a substrate and appropriate blank. In these incubations, blank was achieved by addition of competitive NOS inhibitor, whereas  $\text{Ca}^{2+}$  chelator, ethyleneglycol-bis (36 -aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), served to block  $\text{Ca}^{2+}$ -dependent NOS activity. Thus, the activity of  $\text{Ca}^{2+}$ -dependent enzyme was calculated from the difference between the [ $^{14}\text{C}$ ]citrulline generated from uninhibited samples and samples containing 1 mM EGTA. The activity of the  $\text{Ca}^{2+}$ -independent enzyme was calculated from the difference between samples containing 1 mM EGTA and samples containing both 1 mM EGTA and 1 mM NMMA. The total protein content of tissue supernatant was determined spectrophotometrically (BioRad assay, BioRad, Richmond, California), and final NOS activity was expressed as pmol of citrulline/minutes/mg of protein.

#### ***Immunocytochemical Localization of iNOS Protein in Tissues Analyzed by Light and Electron Microscopy***

Unfixed samples of lungs, intercostal muscles, and spleen were frozen in cryopreservative optimal cutting temperature in OCT (optimal cutting temperature) liquid nitrogen-cooled isopentane, sectioned on a cryostat (20  $\mu\text{m}$ ), and melted directly onto glass slides. Sections were fixed in 10% buffered formalin, endogenous peroxidase activity was blocked with 3%  $\text{H}_2\text{O}_2$  in absolute methanol, and tissue was permeabilized with 0.25% Triton X-100 in PBS. After washing (3  $\times$  5 minutes PBS), 10% normal horse serum in 0.2% BSA was added onto the sections as blocking serum. This was followed by the addition of a complex of a primary (anti-mac NOS, mouse monoclonal antibody against mouse macrophage iNOS; Transduction Laboratories, Lexington, Kentucky) and a secondary horse anti-mouse biotinylated antibody (Dimension Laboratories, Mississauga, Ontario, Canada) (1:50 and 1:200 dilution, respectively). The complex was made by shaking primary and secondary antibody overnight at 4°C, followed by the addition of heat-inactivated normal

mouse serum to a final concentration of 0.2% (v/v) 2 hours before adding the complex to the sections. This procedure, described by Hierck et al (1994), was proven to significantly reduce the high background usually resulting with mouse monoclonal antibodies when applied to mouse tissues. High background is the result of nonspecific binding of secondary anti-mouse Ab to the immunoglobulins normally present in mouse tissues. In the above-mentioned procedure, a complex of primary and secondary antibody is made in a test tube; normal mouse serum is then added to bind to all unbound secondary Ab so that, theoretically, when added, the only possible binding of the complex is to the iNOS antigen present in the tissues. As a negative control, anti-iNOS Ab was replaced with a mouse monoclonal antibody against *Aspergillus niger* glucose oxidase (DAKO; supplied by Dimension Laboratories), an enzyme that is neither present nor inducible in mammalian tissues. Sections were incubated with the complex for 4 hours and washed; avidin-biotin peroxidase complex substrate was then added, followed by diaminobenzidine chromogen treatment. Sections were counterstained with hematoxylin, mounted with aqua-mount, and analyzed for the presence of iNOS protein.

For electron microscopic immunocytochemistry, a slightly modified method described by Xue et al (1996) was used. Cryostat sections immunostained for iNOS on glass slides, as described above, were further processed for viewing in the electron microscope. After incubation in diaminobenzidine substrate, sections were rinsed in distilled water and postfixed with 1% osmium tetroxide. Slides were then placed in a slide transport container, dehydrated in ethanol, and infiltrated with epoxy resin (Polybed 812, Polysciences, Warrington, Pennsylvania). Slides were then removed from the transport containers and placed section-side-up on a horizontal surface. Embedding capsules were placed over the sections and filled with fresh resin; the resin was polymerized overnight in an oven at 60°C. Sections polymerized inside embedding capsules were removed from the surface of the glass slides by repeated plunge-thawing in liquid nitrogen. Ultrathin sections were then prepared and counterstained for 10 seconds in 3% uranyl acetate in 30% alcohol.

#### ***Image Analysis***

Image analysis was performed using an IBM-compatible computer and two computer packages: Jandel Scientific Mocha Image Software and Northern Exposure, version 2.3. Photomicrographs were pre-

pared in a digital darkroom using Adobe Photoshop (Mountainview, California) and Corel Draw software programs (Corel Corporation, Ottawa, Ontario, Canada). Prints were generated with a Tektronic Phaser 440 printer (Wilsonville, Oregon).

### Statistical Analysis

Data were subjected to ANOVA using Microstat Statistics Package (Ecosoft, Inc., Indianapolis, Indiana). A one-way ANOVA test was used for normal distribution, and a Kruskal-Wallis test was used for skewed distributions. Newman-Keuls multiple range test or non-parametric multiple range test was used to further determine which means or sums of ranks were different from one another (Zar, 1974).  $p < 0.05$  was considered significant.

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Effects of  $N^G$ -Nitro-L-arginine Methyl Ester, an Inhibitor of Nitric Oxide Synthesis, on IL-2-Induced LAK Cell Generation *in Vivo* and *in Vitro* in Healthy and Tumor-Bearing Mice<sup>1</sup>AMILA ORUCEVIC AND PEEYUSH K. LALA<sup>2</sup>

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## INTRODUCTION

We had earlier shown that therapy with  $N^G$ -nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthesis, had antitumor and antimetastatic effects in C3-L5 mammary adenocarcinoma-bearing mice. When combined with interleukin-2 (IL-2) therapy, L-NAME augmented antitumor effects of IL-2. In the present study, we tested whether the L-NAME effects were due, at least in part, to a potentiation of antitumor cytotoxicity of host effector cells. We examined the effects of L-NAME on IL-2-induced generation of antitumor cytotoxicity *in vivo* and *in vitro* in splenocytes of healthy and C3-L5 tumor-bearing C3H/HeJ mice, using <sup>51</sup>Cr release assay. IL-2 treatment, *in vivo* or *in vitro*, markedly stimulated splenocyte tumoricidal activity against NK-sensitive (YAC-1) and -resistant (C3-L5) targets, accompanied with an increase in NO production measured in the serum or culture medium. Addition of L-NAME to IL-2 therapy blocked IL-2-induced NO production *in vivo* and improved IL-2-induced splenocyte cytotoxicity as well as tumor regression. Addition of L-NAME *in vitro* also reduced IL-2-induced NO production in the medium and enhanced IL-2-induced cytotoxicity of splenocytes of healthy but not tumor-bearing mice. These results reveal that IL-2-induced increase in NO production *in vivo* causes a suppression of LAK cell activation, which can be overcome by NO inhibition with L-NAME therapy. These findings, combined with our observation that L-NAME can mitigate IL-2-induced capillary leakage in healthy and tumor-bearing mice, suggest that L-NAME could be a valuable adjunct to IL-2 therapy of cancer and infectious diseases. © 1996 Academic Press, Inc.

Interleukin-2 (IL-2)<sup>3</sup> is an important lymphokine which potentiates the functional responses of all cells of the immune system including T cells (1), B cells (2), and natural killer cells (3). Lymphocytes cultured in the presence of IL-2 give rise to lymphokine-activated killer (LAK) cells, by recruitment of primarily NK cells and to a minor extent T lymphocytes (4). LAK cells are capable of killing NK-resistant tumor cells in the absence of prior exposure to tumor antigens and without major histocompatibility locus restriction (5, 6). Functional activation of LAK cells mediating antitumor effects *in vivo* has been demonstrated with IL-2 therapy in numerous species including mice (7, 8) and human (9). Cytolytic function of LAK cells has been shown to be associated with numerous lytic molecules (e.g., perforin) and granule-associated enzymes (e.g., serine esterase) (10).

There is growing evidence to suggest that full therapeutic potential of IL-2 in cancer therapy has not been achieved because of the appearance of suppressor molecules, which can interfere with LAK cell activation. For example, it has been shown in murine models that production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in the tumor-bearing host by tumor cells or host cells (e.g., macrophages) (11, 12) can suppress LAK cell generation by downregulating IL-2 receptors on effector cells (13). Thus, a combination of chronic indomethacin therapy (CIT) with IL-2 markedly improved the antitumor effects of IL-2 therapy, by improving killer cell activation *in situ* (8, 14–16). The possibility also remains that IL-2 therapy itself can induce the production of short-range molecules capable of interfering with LAK cell activation. Nitric oxide (NO) remains a strong candidate.

NO, a short-lived molecule, has been identified as a potent biological mediator (17). It is synthesized in many

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<sup>3</sup> Abbreviations used: IL-2, interleukin-2; LAK, lymphokine-activated killer; NO, nitric oxide; L-NAME,  $N^G$ -nitro-L-arginine methyl ester; NMMA,  $N^G$ -methyl-L-arginine.

mammalian cells from amino acid L-arginine with the help of a family of enzymes named NO synthases (NOS) and plays an active role in many physiological processes such as vasodilation, neural function, and inhibition of platelet aggregation, as well as pathological processes such as inflammation (18–20). The role of NO in tumor immunity remains controversial. First, it has been shown that activated murine macrophages synthesize NO (21), which may partly mediate their cytotoxic activity against tumor cells and bacteria (22–24). Mills *et al.* (24) reported that tumor growth in the mouse peritoneal cavity was associated with a decreased NO production by intratumor macrophages. Similarly, it has been reported that *in vitro* tumoricidal function of IL-2-activated NK cells depends, at least in part, on their NO synthesizing ability, since deprivation of L-arginine in the medium or blocking of NO synthesis with *N*<sup>G</sup>-methyl-L-arginine (NMMA) reduced their killer function (25). In contrast, NO overproduction by rodent macrophages has been shown to suppress activation of T lymphocytes in response to antigen or mitogen (26, 27) and thus may hinder antitumor immune responses of T cells. Indeed, excessive NO production has been implicated in tumor-induced immunosuppression in rats (28). Thus, NO can play a dual role in antitumor defense. While it appears to be an important bioactive component of the cytotoxic pathways of antitumor effector cells, excessive NO release in the microenvironment may be detrimental to their activation pathways.

We have recently begun to examine the contributory role of NO on "capillary leak syndrome," a toxic side effect of IL-2 therapy manifested as severe hypotension and fluid leakage into many organs, including the lungs and body cavities (29). We found that treatment with *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), a potent inhibitor of NO synthesis, ameliorated IL-2-induced capillary leakage in normal (30) as well as tumor-bearing mice (31), whereas treatment with NMMA, another NO inhibitor, was not effective (32). Both agents, however, were found to augment the effects of IL-2 therapy in reducing the tumor burden (31, 33). One explanation of the latter finding was that NO induction by IL-2 interferes with the optimal activation of antitumor effector cells.

The present study was therefore designed to test whether inhibition of NO synthesis with L-NAME could influence IL-2-induced generation of antitumor cytotoxicity *in vivo* or *in vitro* in splenocytes of healthy and C3-L5 mammary adenocarcinoma-bearing C3H/HeJ mice. In addition, the contributory role of macrophages on IL-2-induced NO production *in vitro* was explored by using macrophage-depleted splenocytes.

## MATERIALS AND METHODS

### Mice

C3H/HeJ female mice, 6–7 weeks old, were obtained from the Jackson Laboratories (Bar Harbor, ME). Ani-

mal care was in accord with guidelines of the Canadian Council on Animal Care. Mice were kept on a 12-hr light/dark cycle, fed with a standard mouse chow, and provided with water *ad libitum*.

### Interleukin-2

Highly purified recombinant human IL-2 (lot LQP-046) was kindly provided by the Chiron Corp. (Emeryville, CA). The specific activity was  $18 \times 10^6$  International Units/mg or  $3 \times 10^6$  Cetus Units/mg of IL-2. The lyophilized IL-2 (1.2 mg/vial) was first reconstituted with 1 ml of distilled water and for *in vivo* studies further diluted with RPMI 1640 medium (Gibco BRL, Burlington, ON, Canada) in order to obtain the concentration of 15,000 Cetus Units in 0.1 ml (volume per injection). The reconstituted IL-2 was stored at 4°C for up to 1 day. For *in vitro* studies, IL-2 was further diluted with complete medium in order to obtain the concentration of 1000 Cetus Units/ml/ $4 \times 10^6$  cells. Complete medium was made of RPMI 1640 medium, 10% heat-inactivated fetal calf serum, and 1% antibiotics. Fetal calf serum was obtained from Gibco BRL and antibiotics from Mediatech (Washington, DC). Antibiotics contained 5000 I.U./ml of penicillin and 5000 µg/ml of streptomycin.

### *N*<sup>G</sup>-Nitro-L-arginine Methyl Ester

L-NAME (Sigma Chemical Co., St. Louis, MO) was added to the drinking water to provide concentrations of 0.1, 0.5, and 1 mg/ml of water for *in vivo* experiments. These doses were based on our studies conducted earlier to prevent IL-2-induced capillary leak syndrome in healthy (30) and tumor-bearing mice (31). For *in vitro* experiments, L-NAME was added in concentration of 0.1, 0.5, and 1 mg/ml of complete medium/ $4 \times 10^6$  cells, based on our experience with other *in vitro* studies, in which we have found a dose-dependent reduction in NO production. One milligram per milliliter is the equivalent of 3 mM L-NAME.

### Tumor Cell Line

A spontaneous mammary tumor in a C3H/HeJ mouse which also exhibited lung metastases (34) was the source of a primary transplantable tumor T58, from which the metastatic C3 line had been clonally derived (35). Since the spontaneous lung metastatic ability of the C3 line declined after *in vitro* passages over the years (35), a highly metastatic C3-L5 line was derived from the C3 line by five cycles of *in vivo* selection for spontaneous lung metastasis (14) as follows. C3 cells were transplanted sc into C3H/HeJ mice and allowed to metastasize to the lungs, and cells from the lung micrometastases were then injected sc into fresh C3H/HeJ recipients. This cycle was repeated five times. The C3-L5 line has since maintained its strong metastatic

phenotype in both C3H/HeN (36) and C3H/HeJ (14) strains of mice.

### *Tumor Transplantation*

C3-L5 mammary adenocarcinoma cells ( $2.5 \times 10^5$  in 0.1 ml of RPMI medium) were injected sc in the mammary line near the axilla. In addition to the formation of primary tumors, this procedure was expected to produce micrometastases in the lungs of C3H/HeJ mice within 2 weeks after transplantation (14).

### *Protocols for Immunotherapy*

Healthy or tumor-bearing mice ( $n = 5/\text{group}$ ) were randomly separated into five groups and treated with nothing, IL-2 (10 injections of 15,000 Cetus Units ip every 8 hr), L-NAME (1 mg/ml drinking water), IL-2 + 0.1 mg/ml L-NAME, IL-2 + 0.5 mg/ml L-NAME, or IL-2 + 1 mg/ml L-NAME. Therapies were given in one round. Healthy mice started with L-NAME in drinking water 1 day before IL-2 therapy. Tumor-bearing mice received IL-2 from Day 10 to 13 and L-NAME from Day 9 to 13 after tumor transplantation. Animals were killed 1 hr after last IL-2 injection to measure splenocyte antitumor cytotoxicity and nitrite levels in the serum.

### *Preparation of Single-Cell Suspension of Spleen Cells*

Spleens from healthy or tumor-bearing mice subjected to immunotherapy were pooled (5/each treatment group) and used to isolate effector splenocytes for measurement of LAK cell generation *in vivo*. For *in vitro* LAK cell generation, 30 spleens from healthy or 30 spleens from tumor-bearing mice (14 days after tumor transplantation) were used to isolate effector splenocytes. A single-cell suspension was prepared by homogenization using a glass homogenizer. Pooled cells were resuspended in RPMI 1640, and density gradient centrifugation with sterile modified Ficoll-Paque (1.5 g Ficoll 400/100 ml Ficoll-Paque; Pharmacia, Dorval, PQ, Canada) was used to remove red blood cells and dead cells. Mononuclear cells from the medium-Ficoll-Paque interface were collected, washed, counted, and tested for viability.

### *Generation of LAK Cells in Vitro*

Unfractionated or splenocytes depleted of plastic adherent macrophages (12) from healthy or tumor-bearing mice ( $n = 30$ ) were plated in six-well plates (Corning Glass Works, Corning, NY). Cell input was  $20 \times 10^6$  cells in 5 ml of complete medium alone or complete medium containing 1,000 Cetus Units of IL-2/ml of medium or a combination of IL-2 and different concentrations of L-NAME (0.1, 0.5, or 1 mg/ml of medium). Cells were cultured in duplicate for each experimental condition for 4 days and were used as effector cells to measure tumoricidal activity. In some experiments, cells

were cultured under the same conditions as above, but for 3 days only. Cells were then washed and resuspended in complete medium alone or medium containing IL-2 only, without addition of L-NAME, and incubated for the following 24 hr. This protocol was intended to dissociate the possible requirement of NO production by effector cells during their later phase of activation for their acquisition of killer activity, from the possible suppressive role of NO during the early activation phase of lymphocyte proliferation. Culture medium was collected and kept frozen at  $-20^\circ\text{C}$  for measurement of nitrite levels [final metabolic product of NO production under the culture conditions (37, 38)].

### *Measurement of Splenocyte Antitumor Cytotoxicity*

Cytotoxic activity of *in vivo* or *in vitro* IL-2-activated splenocytes against a NK-sensitive murine tumor line (YAC-1 lymphoma, American Type Culture Collection, Rockville, MD) and a NK-resistant murine tumor line (C3-L5 adenocarcinoma) was measured by  $^{51}\text{Cr}$  release assay (8). Various effector: target ratios (25, 50, and 100:1) were used in triplicate or quadruplicate employing tumor targets labeled for 1 hr with 0.2 mCi of  $^{51}\text{Cr}$  in sodium chromate (Amersham Canada Limited, Oakville, ON, Canada) per  $10^7$  target cells.

### *Measurement of NO Production*

Samples of serum were collected after the end of treatments to measure  $\text{NO}_2^-$  and  $\text{NO}_3^-$ , the principal metabolites of NO (38, 39). Culture media from wells used for *in vitro* generation of LAK cells were collected at the end of the incubation period and kept frozen at  $-20^\circ\text{C}$ , until assayed for  $\text{NO}_2^-$  levels. Griess reagent (40) was used for measurement of  $\text{NO}_2^-$  and cadmium filings for conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  (41).

### *Measurement of the Size of the Primary Tumors*

The size of the primary tumor was measured with calipers on Day 9 and Day 13, by recording the maximum and minimum diameters. Tumor volumes were then calculated as  $0.52a^2b$ , where  $a$  and  $b$  are the minimum and maximum diameters (42). The tumor volume was represented in cubic millimeters.

### *Statistical Analysis*

The Microstat statistics package (Ecosoft, Inc., Indianapolis, IN) was used in the analysis of collected data. One-way ANOVA was used for normal distributions and the Kruskal-Wallis test for nonnormal distributions. The Newman-Keuls test and the nonparametric multiple range test were used, respectively, to determine which means or sums of ranks differed significantly ( $P < 0.05$ ) from one another (43).

While comparing antitumor cytotoxicity of effector cells (*in vivo* or *in vitro* experiments) under different

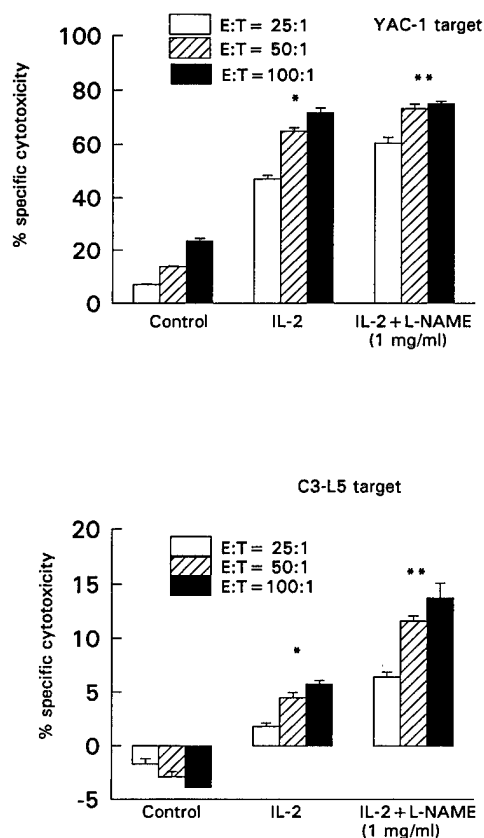


conditions, the results obtained from triplicate or quadruplicate determinations at all effector:target ratios were combined for each group, so that significant differences in overall cytotoxicity could be identified between groups (15).

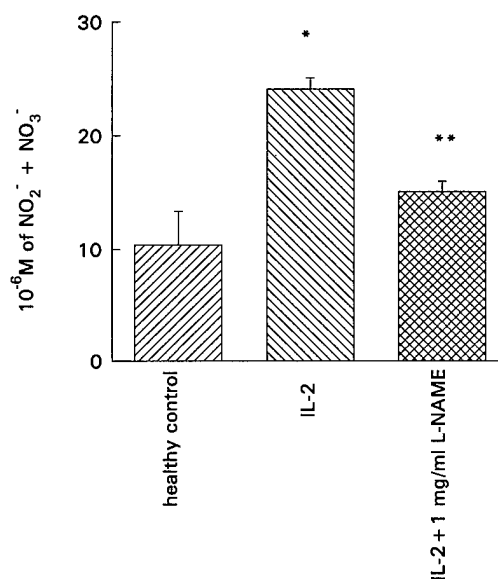
## RESULTS

### Generation of Antitumor Cytotoxicity *in Vivo* in Splenocytes of Healthy Mice

Splenocyte cytotoxicity against both NK-sensitive and NK-resistant targets was markedly ( $P < 10^{-8}$ ) increased after IL-2 treatment of healthy mice. Addition of L-NAME (via drinking water) significantly ( $P < 0.05$ ) enhanced this cytotoxicity further (Fig. 1). The effect was more pronounced ( $P < 0.01$ ) in the case of the C3-L5 (NK-resistant) target. L-NAME therapy



**FIG. 1.** *In vivo* killer cell generation in healthy mice. Data represent means  $\pm$  SE (every effector:target ratio done in triplicate). Cytotoxicities at all effector:target ratios were combined in identifying significant ( $P < 0.05$ ) differences resulting from different experimental conditions in this and other figures on cytotoxicity. \*IL-2 therapy significantly ( $P < 10^{-8}$ ) improved splenocyte cytotoxicity (all three effector:target ratios combined) against NK-sensitive and NK-resistant targets. \*\*Addition of L-NAME therapy significantly ( $P < 0.05$ ) enhanced IL-2-induced splenocyte cytotoxicity (all three effector:target ratios combined) against NK-sensitive and NK-resistant targets.



**FIG. 2.**  $\text{NO}_3^-$  levels in the serum of healthy mice, measured as  $\text{NO}_2^-$  after reduction. Data represent means  $\pm$  SE ( $n = 3-5$ , each done in duplicate). \*IL-2 therapy significantly ( $P < 0.01$ ) increased  $\text{NO}_2^- + \text{NO}_3^-$  levels in the serum, measured at the end of the treatment, compared to untreated control animals. \*\*Addition of 1 mg/ml of L-NAME in drinking water significantly ( $P < 0.01$ ) abrogated IL-2 therapy-induced rise in  $\text{NO}_3^-$  levels in the serum measured at the end of the treatment.

alone did not have any effect on NK activity (data not presented).

### NO Production *in Vivo* in Healthy Mice

IL-2 therapy induced a significant ( $P < 0.01$ ) increase in NO production measured in the serum. This was significantly ( $P < 0.01$ ) abrogated by addition of L-NAME therapy. (Fig. 2).

### Generation of Antitumor Cytotoxicity *in Vivo* in Splenocytes of Tumor-Bearing Mice

IL-2 treatment of tumor-bearing mice significantly ( $P < 0.01$ ) increased splenocyte cytotoxicity against both NK-sensitive and -resistant targets. Increasing doses of orally administered L-NAME significantly ( $P < 0.01$ ) enhanced this cytotoxicity further, reaching a plateau with a dose of 0.5 mg/ml of drinking water (Fig. 3). L-NAME alone did not have any effect on NK activity.

### NO Production *in Vivo* in Tumor-Bearing Mice

IL-2 therapy, again, caused a significant ( $P < 0.05$ ) rise in the serum NO level, which was mitigated ( $P < 0.05$ ) by the addition of L-NAME therapy (data not presented), as reported by us earlier (31).

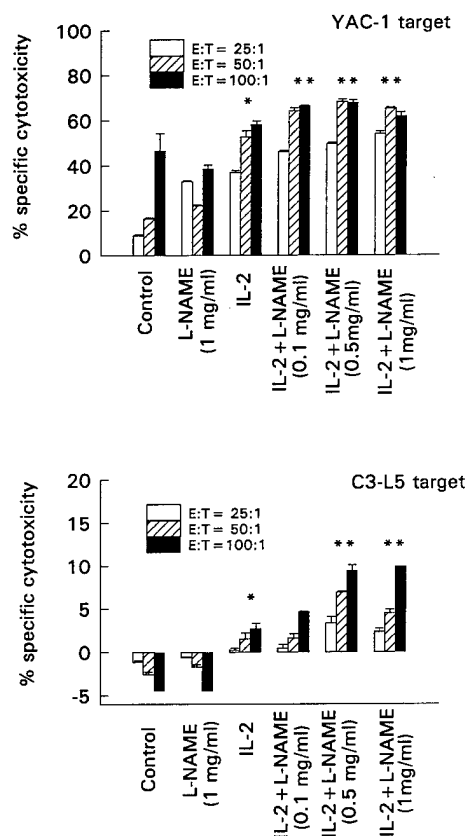
### Tumor Growth during IL-2 and L-NAME Therapy

All therapies significantly ( $P < 0.001$ ) reduced the growth rate of the primary tumors as given by the

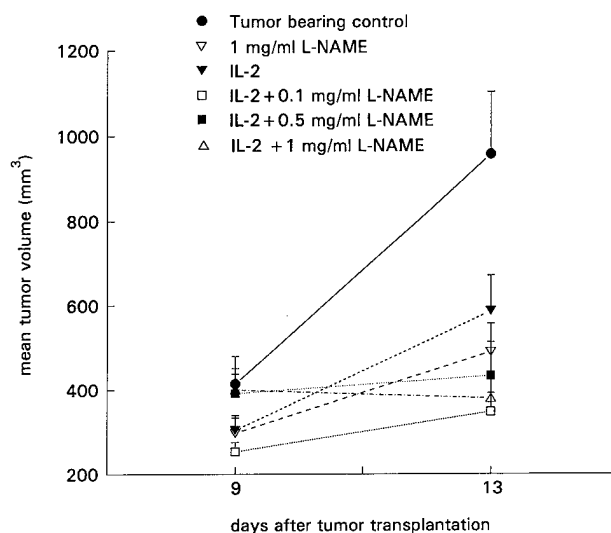
changes in tumor volume between Days 9 and 13. The responses seen with IL-2 + L-NAME (at various doses) were significantly ( $P < 0.05$ ) greater than with IL-2 alone (Fig. 4), confirming our data reported earlier that addition of L-NAME had a beneficial effect on IL-2-mediated reduction of primary tumor growth (31).

#### Generation of Antitumor Cytotoxicity *in Vitro* in Splenocytes of Healthy Mice

L-NAME addition *in vitro* significantly ( $P < 0.05$ ) increased IL-2-induced cytotoxicity of unfractionated splenocytes against the NK-resistant C3-L5 target (Fig. 5, top) and did not have any influence on IL-2-induced cytotoxicity against the NK-sensitive YAC-1 target (data not presented), in the 4-day incubation experiment. Macrophage depletion from the splenocytes caused a minor but significant ( $P < 0.05$ ) improvement of LAK cell activity with IL-2 alone (Fig. 5,



**FIG. 3.** *In vivo* killer cell generation in tumor-bearing mice. Data represent means  $\pm$  SE (every effector:target ratio done in triplicate). \*IL-2 therapy alone significantly ( $P < 0.01$ ) improved antitumor cytotoxicity of splenocytes (all three effector:target ratios combined) against both NK-sensitive and NK-resistant targets. \*\*Addition of 0.5 or 1 mg/ml of L-NAME significantly ( $P < 0.01$ ) enhanced IL-2-induced splenocyte cytotoxicity (all effector:target ratios combined) against NK-sensitive and NK-resistant targets. This was also seen with addition of 0.1 mg/ml L-NAME in the case of NK-sensitive targets ( $P < 0.05$ ).



**FIG. 4.** Growth of the primary tumor during IL-2 and L-NAME therapy, as given by the mean tumor volume on Days 9 and 13. Data represent means  $\pm$  SE ( $n = 14-24$ ). L-NAME alone, IL-2 alone, or IL-2 in combination with any dose of L-NAME significantly ( $P < 0.001$ ) reduced the growth of the primary tumor. Reduction of tumor growth rate (between Days 9 and 13) with IL-2 + L-NAME (at any of the dosages presented) was significantly ( $P < 0.05$ ) greater than with IL-2 alone.

bottom vs top). Addition of L-NAME (0.1 or 1 mg/ml of medium) still led to a minor but significant ( $P < 0.05$ ) improvement of LAK cell activity (Fig. 5, bottom).

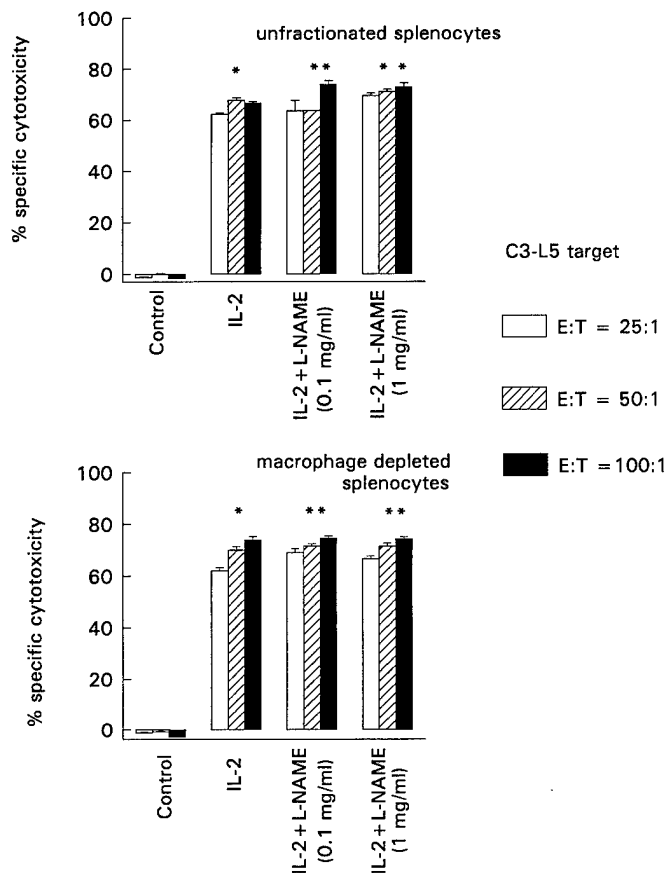
When L-NAME was excluded for the last 24 hr from the incubation medium, there was no significant difference between the cytotoxicity generated with IL-2 alone and the cytotoxicity generated with IL-2 + L-NAME for both unfractionated and macrophage-depleted splenocytes (data not presented).

#### Generation of Antitumor Cytotoxicity *in Vitro* in Splenocytes of Tumor-Bearing Mice

Exposure to IL-2 caused a significant stimulation of antitumor cytotoxicity *in vitro* in splenocytes of tumor-bearing mice. Addition of L-NAME (4 days of incubation) *in vitro* did not, however, significantly influence IL-2-induced cytotoxicity of unfractionated or macrophage-depleted splenocytes (data not presented).

#### NO Production *in Vitro* by Cultured Splenocytes from Healthy or Tumor-Bearing Mice

NO production (as given by the nitrite levels) during 4 days of culture of splenocytes alone from healthy or tumor-bearing mice was low. NO production was significantly ( $P < 0.001$ ) increased when splenocytes from healthy mice or tumor-bearing mice were incubated with IL-2. However, IL-2-induced increase in NO production in splenocytes from tumor-bearing mice was markedly ( $P < 0.001$ ) higher than in splenocytes from



**FIG. 5.** *In vitro* killer cell generation of unfractionated or macrophage-depleted splenocytes from healthy mice against NK-resistant C3-L5 targets. Data represent means  $\pm$  SE (each effector:target ratio done in quadruplicate). \*Addition of IL-2 significantly ( $P < 10^{-7}$ ) induced LAK cell activity in unfractionated (top) or macrophage-depleted (bottom) splenocytes. \*\*Addition of L-NAME (0.1 or 1 mg/ml) significantly ( $P < 0.05$ ) increased LAK cell activity (all effector:target ratios combined) of unfractionated (top) or macrophage-depleted splenocytes (bottom).

healthy mice (Fig. 6, top vs bottom). Addition of L-NAME significantly ( $P < 10^{-5}$ ) reduced the NO levels in both cases, reducing it to the control level at the dose of 1 mg/ml (Fig. 6, top and bottom).

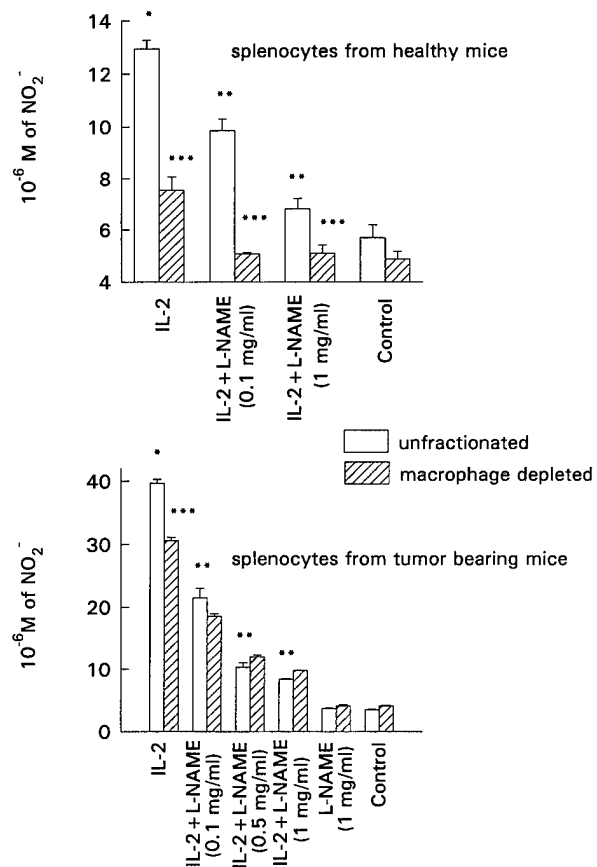
Macrophage depletion significantly ( $P < 0.05$ ) reduced the nitrite levels in cultures incubated with IL-2 and addition of L-NAME reduced them to the control levels, but only in splenocytes from healthy mice (Fig. 6, top).

## DISCUSSION

The present study revealed that inhibition of NO synthesis with L-NAME significantly enhanced IL-2-induced activation of antitumor cytotoxicity of splenocytes *in vivo* in healthy as well as tumor-bearing mice and *in vitro* in healthy mice.

The immunopotentiating effect of L-NAME on IL-2-

induced LAK cell generation *in vivo* in healthy as well as in tumor-bearing mice is a novel finding. We demonstrated earlier that IL-2-induced rise in NO production was directly related to the severity of IL-2-induced capillary leak syndrome (toxic side effect of IL-2 therapy) in healthy mice (30). Inhibition of NO synthesis with L-NAME reduced the severity of IL-2-induced capillary leakage in healthy and tumor-bearing mice. Furthermore, L-NAME therapy alone reduced tumor growth and metastases and in combination with IL-2 augmented early antitumor effects of IL-2, as reported before (31) and confirmed here. Thus, addition of L-NAME to IL-2 therapy provides the dual benefit of reducing IL-2 toxicity and increasing its therapeutic efficacy, both of which were likely due to a reduction of IL-2-induced NO production. Present results, in tumor-free or tumor-bearing mice, reveal that enhanced NO



**FIG. 6.** Nitrite production during 4 days of culture of splenocytes. Data represent means  $\pm$  SE ( $n = 4$ ). \*NO production was significantly ( $P < 0.001$ ) increased when splenocytes from healthy (top) or tumor-bearing mice (bottom) were incubated with IL-2. \*\*Addition of L-NAME significantly ( $P < 10^{-5}$ ) reduced IL-2-induced rise in NO production from splenocytes of healthy or tumor-bearing mice. \*\*\*Macrophage depletion significantly ( $P < 0.05$ ) reduced the nitrite levels in cultures incubated with IL-2 and addition of L-NAME reduced them to the control levels, but only in splenocytes from healthy mice (top).

production during IL-2 therapy, irrespective of the presence of the tumor, interferes with optimal LAK cell activation *in vivo*. L-NAME, by blocking NO production, removes this interference, leading to an improvement of antitumor effects of IL-2 therapy.

*In vitro* results in the present study were in accord with the *in vivo* results in healthy mice. We showed that L-NAME significantly increased IL-2-induced LAK cell activity of unfractionated or macrophage-depleted splenocytes. Similar to the results seen *in vivo*, we also showed that L-NAME blocked IL-2-induced NO production in the medium, in a dose-dependent manner. Thus, it is evident that IL-2-induced NO production was responsible for suboptimal LAK cell activation with IL-2. Macrophages were shown to be the major (but not exclusive) source of IL-2-induced NO production in the splenocyte culture, since macrophage depletion also reduced NO levels in the medium. This finding is in agreement with reports that macrophages after appropriate induction are the major iNOS-expressing cell population in the body (22). Small, but discernible L-NAME effects even after macrophage depletion may have been due to the residual macrophage contamination or some NO production by lymphoid cells after exposure to IL-2.

In the case of tumor-bearing mice, addition of L-NAME did not further stimulate LAK cell activation of IL-2-treated splenocytes *in vitro*, in spite of the fact that NO production in the medium was reduced in the presence of L-NAME and that L-NAME improved IL-2-induced LAK cell generation *in vivo*. The differences between *in vivo* and *in vitro* results may be due to the presence of additional cells in tumor-bearing mice, which were stimulated to produce NO by IL-2 therapy. Indeed, endothelial cells of tumor vasculature (44) as well as macrophages within the tumor have been reported to express iNOS (45). Furthermore, we cannot exclude the possibility that L-NAME effects would have been discernible when added to a lower concentration of IL-2 than that employed in the present study for LAK cell generation.

Previous reports on the role of NO on IL-2-induced LAK cell generation *in vitro* from healthy subjects remain in conflict. Kilbourn *et al.* (46) showed that IL-2-induced LAK cell proliferation and tumoricidal activity of canine lymphocytes toward a canine glioblastoma target were unaffected when NO production was blocked with L-NMMA (another NO inhibitor). Juretic *et al.* (47) reported that L-NMMA suppressed IL-2-induced LAK cell generation from rat or murine splenic cells only when added in culture together with IL-2. The effect was lost when L-NMMA was added a day after IL-2, suggesting that NO might be important in early activation of LAK cells. This group also found that the observed L-NMMA action was species specific, since L-NMMA addition did not affect generation of LAK cells from human peripheral blood or spleen

mononuclear cells. Cifone *et al.* (25) showed that tumoricidal activity of IL-2-induced LAK cells from rat splenocytes was reduced when cytotoxicity assays were performed in the presence of L-NMMA or in arginine-free medium, implying that NO might be important for the effector phase of LAK cell killing. In our present study, we isolated effector cells from healthy or tumor-bearing C3H/HeJ female mice, incubated cells for 4 days in media containing IL-2 or IL-2 + L-NAME, and performed cytotoxicity assays in media free from IL-2 and L-NAME. Removal of L-NAME for the last 24 hr of culture did not influence our results. We suggest that culture conditions as well as species differences may account for the differences in the *in vitro* results among previous (25, 46, 47) and present studies with healthy animals and that our *in vivo* results which were similar in healthy and tumor-bearing mice offer more reliable information on the role of NO on LAK cell activation.

In summary, we have shown that IL-2-induced increase in NO production *in vivo* interferes with LAK cell activation, which can be overcome with L-NAME therapy. Augmentation of early antitumor effects of IL-2 with addition of L-NAME therapy reported by us elsewhere and confirmed here is partly due to this L-NAME action. This provides at least one reason for the beneficial effects of adding L-NAME to IL-2 therapy in reducing the tumor burden (31). These findings, combined with our observation that L-NAME can also mitigate IL-2-induced capillary leakage in healthy (30) as well as in tumor-bearing mice (31), suggest that L-NAME could be a valuable adjunct to IL-2 therapy of cancer and infectious diseases.

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